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(54) Title: HIV-1 ENVELOPE MUTEINS LACKING HYPERVARIABLE DOMAINS (57) Abstract HIV-1 envelope muteins are provided comprising deletions within the hypervariable domains of the polypeptides. Methods of using these proteins in immunoassay and to elicit antibody production are also disclosed, as well as materials and methods useful for producing the muteins by recombinant DNA technology.		

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HIV-1 ENVELOPE MUTEINS
LACKING HYPERVARIABLE DOMAINS

10 Technical Field

The present invention is directed to novel analogs (muteins) of the envelope proteins from human immunodeficiency virus type 1 (HIV-1), methods of making the analogs, DNA sequences encoding the analogs, and
15 methods of using the analogs, for example, in immunoassays and vaccine compositions.

Background

HIV-1, and a recently identified related virus
20 named HIV-2, are the known causative agents of Acquired Immune Deficiency Syndrome (AIDS). Many isolates of HIV-1 have been identified and sequenced. A striking feature of these independent isolates is substantial genomic and amino acid variation, centered particularly in the
25 envelope gene and proteins. This variation among HIV-1 isolates has important implications for both AIDS diagnostics and potential vaccines.

Starcich et al., (1986) Cell 45:637-648, reports on the genetic variation in five independent HIV-1
30 isolates, as well as variations in deduced amino acid sequences. Both conserved and variable regions were observed.

Coffin, (1986) Cell 46:1-4, is a review directed to the variation in the envelope of HIV-1, and the pos-
35 sible mechanism which brings about the variation. Coffin

hypothesized that the highly variable domains, termed "hypervariable" domains, are masking epitopes within the conserved domains from being available for neutralizing antibodies or cell-mediated immune responses. It is concluded that vaccination strategy for HIV-1 should be directed towards developing the ability to provoke an immune response directed against the conserved regions of the envelope despite the presence of masking variable domains.

Modrow et al., (1987) J. Virol. 61:570-578, is also directed to comparison of the amino acid sequences of various HIV-1 isolates. Computer analysis was employed to predict epitopes in the envelope protein, and it was found that the majority of predicted epitopes were located in the hypervariable regions. See, e.g., Figure 1 and Tables 1 & 2, incorporated herein by reference.

Hahn et al., (1986) Science 232:1548-1553, discloses the sequence variations in a series of HIV-1 isolates from a single individual, particularly in the envelope. See also Saag et al., (1988) Nature 334:440-444; Fisher et al., (1988) Nature 334:444-447.

Rusche et al., (1988) Proc. Natl. Acad. Sci. USA 85:3198-3202, discloses that a short peptide having the sequence of an HIV-1 isolate in the third hypervariable domain of the envelope protein was able to absorb isolate-specific neutralizing antibodies from antisera, and that another group was able to elicit isolate-specific neutralizing antibodies by immunization with a peptide from the same domain. It is suggested that since this neutralizing epitope is found in one of the hypervariable domains, that a possible vaccination strategy is to prepare a subunit antigen made up of the critical epitope from multiple isolates. See also Looney et al. (1988) Science 241:357-359.

Goudsmit et al., (1988) Proc. Natl. Acad. Sci. USA 85:4478-4482, reports on the neutralizing ability of antibodies raised in chimpanzees infected with various HIV-1 isolates, and a rabbit immunized with an envelope subunit. The epitope identified by Rusche et al. is reported to be an isolate-specific neutralizing epitope, and that it is immunodominant in HIV-1 chimpanzees.

Due in large part to the generally accepted view that attenuated or killed virus vaccines for AIDS are not feasible, the primary focus for the development of vaccines has been the subunit antigens. Several groups have suggested that short oligopeptides from the envelope domain would make suitable subunit antigens. Other groups are pursuing live recombinant virus vaccines, natural or recombinant viral polypeptide vaccines, or anti-idiotypic vaccines. See, e.g., Koff et al., (1988) Science 241:426-432 (and references cited therein).

A continuing need exists to develop new and better HIV-1 envelope analogs, as well as polypeptides that avoid isolate-specific immune interactions for use in diagnostics and as potential vaccines.

Summary of the Invention

The present invention is directed to HIV-1 envelope analogs (muteins) comprising the constant domains of gp120env or gp160env, but lacking at least one epitope from a hypervariable domain. Despite the reports in the literature suggesting the importance of the epitopes in the hypervariable domains (e.g., immunodominant and/or neutralizing), it has surprisingly been discovered that the muteins of the present invention are useful as diagnostic reagents exhibiting at least as great or greater reactivity to antibodies raised against diverse isolates, and as antigens in raising nonisolate-specific antibodies upon immunization of a mammal.

In one embodiment, the present invention comprises an improved analog of HIV-1 gp120env or gp160env wherein the improvement comprises the deletion of at least one epitope within a hypervariable domain, while retaining
5 the domains conserved among HIV-1 isolates.

In another embodiment, the present invention is directed to a polypeptide comprising epitopes bound by antibodies to HIV-1 gp120env or gp160env and an amino acid sequence according to the formula:
10

C1-V1-V2-C2-V3-C3-V4-C4-V5-C5

wherein:

C1 is an amino acid sequence substantially
15 homologous to Ser29 through Cys130 of HIV-1 SF2;

C2 is an amino acid sequence substantially homologous to Cys199 through Leu291 of HIV-1 SF2;

C3 is an amino acid sequence substantially homologous to Ser366 through Cys387 of HIV-1 SF2;

20 C4 is an amino acid sequence substantially homologous to Cys415 through Gly456 of HIV-1 SF2;

C5 comprises an amino acid sequence substantially homologous to Phe466 through Arg509 or Leu855 of HIV-1 SF2;

25 V1 is an amino acid sequence of 0 to a maximum of about 30 residues;

V2 is an amino acid sequence of 0 to a maximum of about 50 residues;

30 V3 is an amino acid sequence of 0 to a maximum of about 90 residues;

V4 is an amino acid sequence of 0 to a maximum of about 30 residues; and

V5 is an amino acid sequence of 0 to a maximum of about 10 residues;

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with the proviso that at least one of the V domains selected from the group consisting of V1, V2, V3, V4 and V5 contains no more than about one-third of the said maximum number of residues for the V domain.

5 Still another embodiment of the present invention is directed to a polypeptide analog of HIV-1 gp120env or gp160env comprising (a) about 300 to about 850 amino acid residues in length; (b) constant domains, in the N-terminal to the C-terminal direction, Ser29-Cys130 of HIV-10 1 SF2 (C1), Cys199-Leu291 of HIV-1 SF2 (C2), Ser366-Cys387 (C3), Cys415-Gly456 (C4) of HIV-1 SF2, and Phe466-Arg509 or Phe466-Leu855 of HIV-1 SF2 (C5), or domains substantially homologous to said C1, C2, C3, C4 or C5; and (c) the intervening domains, if any, located between said15 constant domains comprising sequences found between substantially homologous constant domains in native HIV-1 gp120env, with the proviso that at least one of said intervening domains between said constant domains is either missing or missing an epitope.

20 The present invention is also directed to an immunoassay for the detection of antibodies to HIV-1 comprising: (a) providing a liquid sample to be tested for the presence of anti-HIV-1 antibodies; (b) contacting said sample with a polypeptide as described above under25 conditions whereby any anti-HIV-1 antibodies present in said sample may bind to an epitope to provide sample-contacted polypeptide; and (c) detecting any antibody bound to said sample-contacted polypeptide.

In yet another embodiment, the present invention30 is directed to a method of selectively raising antibodies in a mammal to epitopes in the constant domains of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env comprising administering to said mammal a polypeptide as described above, whereby antibodies to said polypeptide35 are produced in response to said administration.

The present invention is also directed to a composition useful in such method comprising the polypeptide described above in combination with a pharmaceutically acceptable carrier.

5 The present invention is also directed in another embodiment to a DNA sequence encoding the above polypeptide, as well as a cellular host comprising the DNA sequence under the control of transcriptional and translational control sequences whereby the polypeptide
10 encoded by the DNA sequence is expressed by the cellular host. The present invention is also directed to methods of producing the above polypeptide by growing a culture of the above cellular host under conditions whereby the polypeptide encoded by the above DNA sequence is
15 expressed, and recovering the polypeptide from the culture.

These and other embodiments of the present invention will be apparent to those of ordinary skill in the art.

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Brief Description of the Figures

Figure 1 is a schematic diagram of the wild-type gp120env gene from HIV-SF2.

25 Figure 2 shows the alignment of amino acid sequences for various HIV-1 isolates, including SF2, with the constant and variable domains indicated. Potential N-linked glycosylation sites, for the HXB2 sequence only, are indicated by "[]"; cysteine residues have "*" above them.

30 Figure 3 is a restriction map of mammalian expression vector pSV7dARV120tpa.

Figure 4 is a restriction map of mammalian expression vector pCMV6ARV120tpa.

35 Figure 5 is a restriction map of yeast expression vector pHL15.

Figure 6 contains graphs showing the result of an ELISA testing the reactivity of HIV-1 envelope muteins of the present invention and controls against a North American serum panel.

5 Figure 7 contains graphs showing the result of an ELISA testing the reactivity of HIV-1 envelope muteins of the present invention and controls against an African serum panel.

10 Figure 8 shows the results of an ELISA using sera from guinea pigs immunized with envelope muteins according to the present invention or various controls.

15 Figure 9 shows the results of an ELISA using sera from goats immunized with envelope muteins according to the present invention or various controls.

Detailed Description

HIV-1 is a known virus, of which many isolates have been observed. See, e.g., Levy et al., (1984) Science 225:840; Barre-Sinoussi et al., (1983) Science 220:868; Popovic et al., (1984) Science 224:497; AIDS: Papers from Science, 1982-1985 (R. Kulstad ed. 1986); Current Topics in AIDS (M. Gottlieb et al. eds. 1987). The envelope gene of HIV-1 produces a precursor glycoprotein of approximately 160 mw, referred to as gp160 or gp160env. The precursor is cleaved during expression to provided gp120 (gp120env), the major exterior envelope glycoprotein, and gp41 (gp41env), the transmembrane protein. Various HIV-1 isolates have been reported in the literature, including, BH10, PV22, BRU, HXB2, WMJ1, WMJ2, WMJ3, CDC4, HTLV-IIIRF, HTLV-IIIB, Z3, Z6, Z321, MAL, NY5, ELI, JY1, LAV1A, and HAT3. Of particular interest to the present invention is HIV-1 isolate SF2, originally designated ARV2. See, e.g., Levy, U.S. Patent No.

4,716,102; Levy et al., (1984) Science 225:84; EPO Pub. No. 181,150.

The conserved and hypervariable domains of HIV-1 envelope proteins have been described previously. See, 5 e.g., Modrow et al., supra. Figure 1 herein is a schematic diagram showing the location of the five hypervariable regions (V1, V2, V3, V4 and V5) and the five constant regions (C1, C2, C3, C4 and C5) in the envelope gene from HIV-1 SF2. Various deletions encompassing part 10 or all of a hypervariable domain are also shown (D1, D2, D3, D4 and D5). Two regions of predominantly hydrophobic amino acids are denoted by dark shading, where the transmembrane anchor has been labeled TM. The signal sequence is highlighted at the N-terminus, as well as the 15 processing site separating gp120env (positions 1-1527) from gp41env (positions 1528-2565).

Hypervariable domains are characterized by a substantial lack of homology (e.g., as low as 10%) among independent HIV-1 isolates. Furthermore, there is a 20 substantial variation in length among the hypervariable domains from various isolates due to the prevalence of insertion and deletion mutations. Thus, these regions cannot be characterized from one isolate to the next by having any substantial degree of amino acid sequence 25 homology, and can only be assigned an approximate length. The primary characterization of hypervariable domains is their location within the envelope glycoprotein and their presumed tertiary structure (i.e., loops). The conserved or constant domains, as well as all 18 cysteines in the 30 envelope, are highly conserved. Corresponding hypervariable domains are found to be located identically relative to surrounding constant domains and cysteines from one isolate to the next. Furthermore, the tertiary structure of the hypervariable domain appears to be highly 35 conserved; e.g., two nonhomologous hypervariable domains

from different HIV-1 isolates will usually both exhibit the same three-dimensional conformation, such as an exposed loop. Thus, hypervariable domains from new HIV-1 isolates can be readily identified by sequencing the new isolates and comparing the sequence to known HIV-1 sequences so that the conserved domains and cysteines are aligned. See, e.g., Modrow et al., supra and Figure 2 herein.

The location of the various domains, both constant and hypervariable, will be described hereinafter with reference to the sequence and numbering of the HIV-1 SF2 isolate. It is to be understood that this is for convenience only; the invention is not limited to analogs containing only HIV-1 SF2 sequences, but also encompasses analogs employing corresponding domains or sequences from other isolates. A domain from another HIV-1 isolate envelope protein can be easily identified as corresponding to an SF2 sequence by those of ordinary skill in the art by alignment of the conserved domains and the 18 cysteines of both isolates. Such an alignment is shown in Figure 2, where the corresponding sequences of 15 HIV-1 isolates are aligned. The cysteine residues are marked with an asterisk, and potential N-linked glycosylation sites for the HXB2 isolate are indicated by "[]". The cleavage site for the signal peptide and for the mature gp120env and gp41env proteins are shown, along with the constant domains, C1-C5. Specific deletions, D1-D5, are also shown in the figure.

The constant domains of the SF2 isolate are as follows: C1, Ser29-Cys130; C2, Cys199-Leu291; C3, Ser366-Cys387; C4, Cys415-Gly456; and C5, which is Phe466-Arg509 for gp120env analogs, or Phe466-Leu855 for gp160env analogs. Since these domains are highly conserved among HIV-1 isolates, the corresponding sequences from isolates other than SF2 will be substantially homologous to these

SF2 domains; i.e., a minimum of about 70-75% amino acid sequence homology, with certain highly conserved domains exhibiting a minimum of about 80%, or even 85-90% homology. The differences in amino acid sequences found in these conserved domains are generally attributable to point mutations in the nucleic acid sequence, as opposed to the deletion and insertion mutations which typify the differences in hypervariable domains.

As shown in Figures 1 and 2, the hypervariable domains lie between the conserved domains, C1-C5. Not all of the amino acids in these intervening domains, however, comprise hypervariable regions. Indeed, due to the heterogeneity among HIV isolates in the hypervariable domain, it is not feasible to generally define precise limits of the hypervariable domain. Thus, for the convenience of describing the present invention, the domains between the constant domains will be referred to as "variable" or "intervening" domains, and it will be understood that they may comprise both hypervariable regions as well as less variable, yet not highly conserved, sequences. Thus, the analogs of the present invention can be described schematically according to the following formula:

C1-V1-V2-C2-V3-C3-V4-C4-V5-C5 (I)

C1-C5 and V1-V5 in formula I are defined as described above in the Summary of the Invention. V1-V5 consist of the variable or intervening domains which contain hypervariable regions. The polypeptides of the present invention will contain a deletion in at least one of these variable domains when compared to a native gp120env or gp160env. The deletions will typically be at least about one-third of the variable domain, and oftentimes will consist of a deletion of the entire variable domain.

Furthermore, it is also preferred to delete sequences from more than one of the variable domains, including the deletion of all five of the variable domains in their entirety. Thus, in accordance with the present invention, 5 any of the variable domains, V1-V5, can be anywhere from zero to a maximum number of amino acids in length, the maximum being an approximation of the longest of such domains found in HIV-1 isolates. At least one of the V domains, however, will have deleted therefrom at least 10 one-third of that maximum number of amino acids, or one-third of the length of the corresponding domain from the homologous HIV-1 isolate.

Since the variable or intervening domains of formula I can comprise more than the true hypervariable 15 domains, a selected Vn domain can be expressed by the following formula:

$$S_n-HV_n-S_n' \quad (II)$$

20 wherein n is 1, 2, 3, 4 or 5 (as in V1, V2, etc.), HVn is a hypervariable domain of x amino acid residues in length, and Sn and Sn' are nonhypervariable sequences flanking HV in the Vn domain, the flanking sequences being y and y' residues in length, respectively. In the native protein, 25 the sum of x, y and y' equals the maximum number of residues for Vn in a chosen isolate, and y and/or y' may be zero. As for the analogs of the present invention, the sum of x, y and y' will be anywhere from zero to the maximum number. The actual values for x, y and y' for a 30 particular Vn can be determined by sequence comparison of a representative number of HIV-1 isolates. See, e.g., Figure 2 and Modrow et al., supra.

The following are the intervening domains of the SF2 isolate, which can be used for comparison purposes to 35 determine the corresponding intervening domains in other

HIV-1 isolates. See, e.g., Figure 2. V1 of SF2 is about 24 amino acids in length, encompassing Thr131 through Asn154 and, optionally, Cys155. This C-terminal cysteine is one of the 18 highly conserved cysteine residues found in gp120env. The corresponding domains from independent HIV-1 isolates sequenced to date appears to range from about 22 to about 31 amino acid residues in length. V2 of SF2 spans from Ser156 through Ser198. The corresponding domain in other HIV-1 isolates sequenced to date appears to range from about 39 to about 52 amino acid residues in length. V3 of SF2 spans Asn292 through Glu365, and the corresponding domains in other HIV-1 isolates sequenced to date appears to range from about 88 to about 90 amino acid residues in length. V4 of SF2 spans from Asn388 through Pro414, and the corresponding domains from other HIV-1 isolates appear to range from about 28 to about 33 residues in length. V5 of SF2 spans from Thr457 through Thr463, and corresponding domains from other HIV-1 isolates appear to range from about 10 to about 11 residues in length.

In selecting the areas of the intervening domains for deletions, it is preferred to either select those portions showing hypervariability, or areas known to encode epitopes which elicit a significant immune response in vivo. Examples of such epitopes in variable regions of the SF2 isolate include, without limitation, residues 137-158 (V1), residues 189-209 (V2), residues 300-327 (V3), residues 367-384 (V3), and residues 404-420 (V4). Corresponding epitopes from other isolates are known. Modrow et al., supra. Examples of preferred deletions for SF2, D1-D5, are shown in Figures 1 and 2: Thr131-Asn154 (D1), Ser156-His198 (D2), Thr300-His332 (D3), Asn388-Pro414 (D4), and Thr457-Thr463 (D5). The polypeptides of the present invention may also have deletions from more than one variable domain, for example, V1 and V2; V1, V2 and

V3; V1, V2 and V5; V3, V4 and V5; or V1, V2, V3, V4, and V5.

In a preferred embodiment, the HIV-1 envelope analogs of the present invention have deleted therefrom an epitope found in the intervening domains which is bound by antibodies produced by a mammal infected or immunized with the particular HIV-1 isolate, or its native envelope protein. It is particularly preferred that the deleted epitopes are those which produce isolate-specific immunodominant responses in a mammal. While applicants do not wish to be bound by this theory, it is believed that the deletion of these variable epitopes unmasks epitopes in the conserved domains which then become visible to the immune system by virtue of the absence of the epitopes from the variable domains.

In general, the amino acid sequence according to formula I will range from about 300 to about 850 amino acid residues in length, the actual length not being critical. The sequence of formula I can be contained within a larger polypeptide, for example a fusion protein. Such fusion proteins can include, for example, a fusion between the N-terminal sequence of superoxide dismutase (human or yeast) or beta-galactosidase, where these non-HIV-1 sequences are fused to the N-terminal of the C1 domain. Alternatively, non-HIV-1 sequences could also be fused to the C-terminal of the C5 domain. The C1 domain may also be fused to a signal peptide (e.g., yeast alpha factor, or tpa signal) to provide for secretion of the HIV-1 envelope analog from a cellular host expressing the analog.

The HIV-1 envelope muteins of the present invention can be produced by any suitable method, such as direct peptide synthesis or recombinant DNA expression. The preferred method is to prepare the polypeptides by recombinant DNA techniques.

The methodology for preparing such recombinant polypeptides is within the skill of the art, and the techniques are fully explained in the literature. See, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, Volumes I & III (D.N. Glover, ed., 1985); Oligonucleotide Synthesis: A Practical Approach (M.J. Gate, ed., 1984); Perbal, A Practical Guide to Molecular Cloning (1984). The production of recombinant HIV-1 polypeptides is known in the art. See, e.g., Luciw et al., (1984) Nature 312:760; Sanchez-Pescador et al., (1985) Science 227:484; Hahn et al., (1984) Nature 312:167; Alizon et al., (1984) Nature 312:757; Ratner et al., (1985) Nature 313:636; Muesing et al., (1985) Nature 313:450; Wain-Hobson et al., (1985) Cell 40:9; EPO Pub. Nos. 181,150; 187,041; 227,169; 230,222; and PCT Pub. Nos. WO87/02038; WO87/02989; WO87/04459; WO87/04728. Methods of recombinant expression are also disclosed in commonly owned U.S. patent application Serial No. 138,894, filed 24 December 1987, entitled "Human Immunodeficiency Virus (HIV) Nucleotide Sequences, Recombinant Polypeptides, and Applications Thereof," the disclosure of which is incorporated herein by reference.

To prepare the polypeptides of the present invention by recombinant methods, a DNA coding sequence for the polypeptides must be provided. Such a coding sequence is a DNA sequence that can be transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by and include the translation start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. DNA sequences encoding the polypeptides of the present invention can be prepared either by constructing a synthetic gene from overlapping oligonucleotides, or by site-directed mutagenesis of a sequence encoding native

HIV-1 envelope. See, e.g., Zoller, & Smith (1983) Meth. Enzymol. 100:468-500.

DNA coding sequences for the polypeptides of the present invention can be maintained on a replicon, which
5 is any genetic element (e.g., plasmid, cosmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control. Vectors are replicons such as a plasmid, phage, or cosmid to which a DNA coding sequence may be
10 attached so as to bring about the replication of the attached segment in vivo.

A host cell is then transformed with a DNA construct containing the coding sequence under the control of appropriate regulatory sequences in order to bring
15 about the expression of the DNA coding sequence into the desired polypeptide. Cellular hosts can include, but are not limited to, bacteria (e.g., E. coli, Bacillus subtilis, B. amyloliquefaciens, Salmonella typhimurium, Klebsiella pneumoniae or Erwinia amylovora), yeast (e.g.,
20 Saccharomyces cerevisiae, S. carlsbergensis, S. kluyveri, Kluyveromyces lactis, Pichia, or Schizosaccharomyces), mammalian cells (e.g., CHO cells, COS cells, 293 cells or Xenopus oocytes), and insect cells (e.g., Drosophila embryos or Spodoptera frugiperda). The transformed cel-
25 lular hosts may contain the DNA constructs encoding the polypeptides of the present invention either on an extrachromosomal element, or integrated into the chromosome.

Typically, the DNA coding sequence is placed
30 into an expression cassette which comprises the DNA coding sequence flanked by appropriate regulatory sequences that control transcription initiation and termination within the cellular host. Preferably, the expression cassette contains convenient restriction sites at either end to

permit convenient cloning of the cassette into an appropriate vector for transformation of the cellular host.

Transcription initiation and termination sequences recognized by the cellular host are DNA regulatory regions which flank a coding sequence are responsible for the transcription of an mRNA homologous to the coding sequence which can be translated into the desired polypeptide. Transcription initiation sequences include host promoter sequences, which are DNA regulatory sequences capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. A coding sequence is "under the control" of transcription initiation and termination sequences when RNA polymerase binds the transcription initiation sequences and transcribes the coding sequence into mRNA terminating at the transcription termination sequence, and the mRNA is then translated into the polypeptide encoded by the coding sequence (i.e., expression).

Cellular hosts transformed with appropriate DNA constructs for the expression of polypeptides of the present invention are typically grown in a clonal population under appropriate conditions which bring about the expression of the DNA coding sequence of interest. The appropriate growth conditions will depend upon the cellular host and the transcriptional and translational regulatory sequences employed. Upon expression, the recombinant polypeptide is recovered from the culture by any appropriate method, e.g., gel chromatography, immunoabsorption, or gel electrophoresis.

Polypeptides of the present invention can be used as reagents in immunoassays for detecting the presence in a sample of either anti-HIV-1 antibodies or viral antigen. In an immunoassay for viral antigen, for example, polypeptides according to the present invention

can be labeled and used as a competing antigen in a standard competitive ELISA or radioimmunoassay (RIA).

Configurations for immunoassays for antibodies to HIV-1 envelope vary widely, and the present invention
5 contemplates the use of the polypeptides described herein in any such format. Such formats are well known in the art. See, e.g., Immunoassay: A Practical Guide (P.W. Chan & M.T. Perlstein, eds., 1987); McDougal et al., (1985) J. Immunol. Meth. 76:171-183; U.S. Pat. Nos.
10 4,629,783; 4,281,061; 4,520,113; 4,591,552; 4,134,792 (incorporated by reference herein). Whether the assay format is homogeneous or heterogeneous, and the measurement method is direct or indirect (e.g., competition), all such immunoassays have three common steps. First, a liquid
15 sample suspected of containing the antibodies is provided. This sample is then contacted with polypeptides according to the present invention under conditions which will allow any antibodies having an epitope on the polypeptides to become bound thereto. Finally, there is a detecting step
20 wherein it is determined whether or not any antibody bound to the polypeptide.

A preferred format for an anti-HIV-1 antibody assay is a heterogeneous immunoassay in which the polypeptides of the present invention are immobilized on, for
25 example, a solid support. The selection of the appropriate solid support for immobilization of the polypeptide is conventional and within the skill of the art. In a typical assay, the sample suspected of containing the antibodies is contacted with the immobilized polypeptide
30 and allowed to incubate under the appropriate conditions. The immobilized polypeptide is then separated from the sample and washed to remove any unbound antibody. The detecting step can constitute, for example, contacting the washed, immobilized polypeptide with an antibody that will
35 recognize an epitope located on the anti-HIV-1 antibodies

(i.e., anti-xenogenic), this second antibody being appropriately labeled for detection (e.g., radiolabeled, enzyme conjugated, avidin/biotin). After an appropriate incubation and washing step, the immobilized polypeptide
5 is then assayed for the presence of the labeled second antibody. Alternatively, a competition immunoassay can be used where a labeled reference antibody to the immobilized polypeptide is incubated along with the sample suspected
10 of containing the anti-HIV-1 antibodies, and the presence of such antibodies are determined by measuring a reduction or inhibition of binding of the labeled reference antibody to the immobilized polypeptide. See, e.g., PCT Pub. No. WO87/07957.

Kits suitable for immunodiagnosis and containing
15 the appropriate labeled reagents are constructed by packaging the appropriate materials, including the polypeptides of the present invention and any antibodies used in the assay, in suitable containers along with remaining reagents and materials required by the assay
20 format; e.g., incubation media, wash media, and means for measuring the presence of the analyte (e.g., enzyme-labeled antibodies and enzyme substrate). Other labels useful in the practice of immunoassays according to the present invention include radioisotopes and fluorescing
25 compounds.

Polypeptides according to the present invention can be employed to selectively raise antibodies in a mammal to epitopes found in the constant domains of gp120env or gp160env. For example, parenterally administering
30 polypeptides of the present invention to a mammal will cause an immune reaction in the animal, thereby producing antibodies to epitopes found in the conserved domains. Such antibodies can be recovered to make polyclonal antiserum, or antibody-producing cells recovered for fusion (or another immortalization technique) to produce
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monoclonal antibody-producing cell lines. See, e.g., M. Schreier et al., Hybridoma Techniques (1980); Hammerling et al., Monoclonal Antibodies and T Cell Hybridomas (1981); Kennett et al., Monoclonal Antibodies (1980); U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887. Antiserum produced by the above method will be advantageous in that it will possess a high titer of antibodies which are reactive to all or most HIV-1 isolates. In a similar manner, the screening of hybridomas for non-isolate-specific antibodies will be facilitated by the elimination of some or all of the immunodominant epitopes of the hypervariable regions.

To generate such an antibody response, polypeptides of the present invention are typically formulated with a pharmaceutically acceptable carrier for parenteral administration. The formulation of such compositions, including the concentration of the polypeptide and the selection of the vehicle and other components, is within the skill of the art.

A pharmaceutically acceptable vehicle, suitable for parenteral injection, is usually nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Parenteral vehicles may also take the form of suspensions containing viscosity-enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. The vehicle will also usually contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and tris buffer, while examples of preservatives include thimerosal, m- or c-cresol, formalin and benzyl alcohol. The muteins of the present invention may also be formulated into liposomes for parenteral

administration. Standard formulations will either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a nonliquid formulation, the vehicle may comprise
5 dextrose, human serum albumin, preservatives, etc., to which sterile water or saline could be added prior to administration.

Various adjuvants are known in the art which can also be employed in the vaccine formulations of the
10 present invention; e.g., Freund's adjuvant, avridine, aluminum salts $[Al(OH)_3AlPO_4, Al_2(SO_4)_3]$, $Ca_3(PO_4)_2$, saponin, DDA, Plusonics, oil-in-water emulsions (containing, e.g., avridine, dextran sulfate or vitamin E), water-in-oil emulsions (containing, e.g., polysorbate 8), and
15 muramyl peptides (e.g., di- and tripeptides in carriers such as oil-water emulsions or liposomes). The selection of the appropriate adjuvant and its concentration in the vaccine composition is within the skill of the art.

Many protocols for administering the vaccine
20 composition of the present invention to animals are within the skill of the art. The preferred route of administration is parenteral, particularly intramuscular, although administration may also be intravenous. The concentration of the polypeptide antigen in the vaccine composition
25 is selected so that an effective dose is presented to the host mammal (e.g., primate) to elicit antibodies to the polypeptide's epitopes. Within wide limits, the dosage is not believed to be critical. Typically, the vaccine composition is administered in a manner which will deliver
30 between about 1 to about 1,000 ug of the polypeptide antigen in a convenient volume of vehicle (e.g., about 1-10 ml). Preferably, the dosage in a single immunization will deliver from about 1 to about 500 ug of polypeptide antigen, more preferably about 5-10 to about 100-200 ug
35 (e.g., 10-100 ug). It may also be preferred, although

-21-

optional, to administer a second, booster immunization to the mammal several weeks to several months after the initial immunization. It may be helpful to readminister a booster immunization to the mammal once every several
5 years to maintain high antibody titer.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the claims in any way.

10 EXAMPLES

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The following example describes the construction
15 of DNA sequences encoding HIV-1 SF2 envelope muteins according to the present invention. The hypervariable regions and the corresponding deletions are shown below in Table 1.

20 Table 1
Hypervariable Regions and Deletion Mutants

	<u>Hypervariable Regions</u>	<u>Corresponding Deletion Mutant(s)</u>
25	V1 131 through 154	D1 131 through 154
	V2 156 through 198	D2 156 through 198
	V3 292 through 365	D3 300 through 332
	V4 388 through 414	D4 388 through 414
30	V5 456 through 465	D5 457 through 463

DNA encoding sequences for muteins of the present invention were prepared by mutagenesis of a gp120env SF2 gene. Mutagenesis was achieved employing the
35 procedure described by Zoller and Smith, (1983) Meth.

Enzymol. 100:468-500, modified as described below.

Synthetic DNA mutagenesis and sequencing primers (Table 2) were prepared by automated oligonucleotide synthesis on a silica support as described by Urdea et al., (1983) Proc. Natl. Acad. Sci. USA 80:7461-7465, using N,N-diisopropyl phosphoramidites. Sequencing primers were designed for sequencing by the dideoxynucleotide chain termination method in bacteriophage M13. Sanger et al., (1977) Proc. Natl. Acad. Sci. USA 74:5463. The sequencing primers were designed to be complementary to M13mp8 recombinant templates, of 18 bases in length, and to anneal at a position at least 50 bases away from the mutation locus. Table 2 indicates the oligomers employed for mutagenesis screening and sequencing..

The mutagenesis template contained the entire plasmid pSV7dARV120tPA, which contains the SF2 gpl20env gene coupled to the tPA signal sequence (described below). The plasmid was linearized at its unique PstI site and cloned into the unique PstI site of M13mp8.

Table 2

Mutagenesis Primers

25	D1	5' CCACTCTGTGTTACTTTAAATTGCTGCTCTTTCAATATCACCACAAGC 3'
	D2	5' ATAAAAGGAGAAATAAAAACTGCTGTAACAGATCAGTCATTACACAG 3'
	D3	5' AATGAATCTGTAGCAATTAAGTGTGTAACATTAGTAGAGCACAATGG 3'
	D4	5' GGGGAATTTTCTACTGTTGTAGAATAAAACAAATTATAAACATGTGG 3'
30	D5	5' CTGCTATTAAACAAGAGATGGTGGTGAGGTCTTCAGACCTGGAGGAGGA 3'
	D1+D2	5' CCACTCTGTGTTACTTTAAATTGCTGCTGTAACAGATCAGTCATTACA 3'

DNA Sequencing Primers

D1,D2,D1+D2 5' TAATCAGTTTATGGGATC 3'
 D3 5' CTGTTAAATGGCAGTCTA 3'
 5 D4,D5 5' CAATCCTCAGGAGGGGAC 3'

Screening Probes

D1 5' TTAAATTGCTGCTCTTTC 3'
 10 D2 5' AAAAAGTCTGTAACAGA 3'
 D3 5' AATTAAGTGTGTAACATTA 3'
 D4 5' GGGGAATTTTCTACTGTTGTAGAATAAAACAAATTATAAACATGTGG 3'
 D5 5' ATGGTGGTGAGGTCTT 3'
 15 D1+D2 5' CCACTCTGTGTTACTTTAAATTGCTGCTGTAACAGATCAGTCATTACA 3'

Mutagenesis of M13/pSV7dARV120tPA recombinants was performed using purified templates by annealing and extending the appropriate primer with the Klenow fragment of DNA polymerase I. Individual deletion mutants D1, D2, D3 and D5 were obtained utilizing the appropriate mutagenesis primer in a single round of mutagenesis (see Table 2). Deletion mutant D4 was obtained utilizing the appropriate mutagenesis primer with the annealing and elongation reactions performed at 37°C and in the presence of 125 ug/ml gene 32 protein. Combination deletion mutant D1+D2 was obtained utilizing the appropriate mutagenesis primer using a template derived from deletion mutant D1.

Following transfection of JM101 cells (Zoller and Smith, supra), plaques were grown at a density of 200-1,000/plate and lifted onto filters and screened by hybridization with the appropriate mutagenesis primer or probe (see Table 2).

The DNA sequence of putative positive clones was determined using suitable primers and template preparations. Once the mutagenized locus and flanking segments (i.e., at least 50 bases) were confirmed by DNA sequence analysis, replicative form (RF) DNAs were digested by PstI restriction endonuclease and the entire mutagenized mammalian expression vector pSV7dARV120tPA containing the deletion was recovered. The vector provides an SV40 early promoter and enhancer for expression of SF2 gp120env gene, SV40 polyadenylation site, and an SV40 origin of replication for use of the vector in COS cells.

Following recovery of pSV7dARV120tpa plasmids for wild-type gp120 or for a deletion mutant, the plasmid was digested with SalI to excise the complete gene spliced to the human tPA 5' untranslated sequences and signal sequences. The SalI fragment was subcloned into the unique SalI cloning site of the mammalian cell expression vector pCMV6a. The resulting plasmid was screened to verify the correct orientation of the gene with respect to the promoter and polyadenylation signals, and this plasmid was named pCMV6ARV120tpa for the wild-type gp120 sequences. In cases where the pSV7d vector was not recovered directly as a plasmid, the SalI fragment containing the gene was subcloned from the M13 mutagenesis template clone into pCMV6a.

Combination deletion mutant D4-D5 was obtained utilizing the appropriate D5 mutagenesis primer using a template derived from deletion mutant D4.

Combination deletion mutant pSV7d120D3-D4-D5 was obtained by subcloning the region containing D4-D5 by digestion of M13pSV7d120D4-D5 with MstII and HindIII and insertion of this 334 bp fragment into MstII and HindIII digested pSV7d120D3. The SalI fragment from pSV7d120D3-D4-D5 containing D3-D4-D5 was subcloned into the SalI site of pCMV6a to create pCMV6a120D3-D4-D5.

Combination deletion mutant D1-D2-D5 was obtained by subcloning the region containing D1-D2 by digestion of pCMV6a120D1-D2 with NheI and BglII and insertion of this 540 bp fragment into NheI and BglII digested pCMV6a120D5.

Combination deletion mutant D1-D2-D3-D4-D5 was obtained by subcloning the fragment containing the D1-D2 region by digestion of M13pSV7d120D1-D2 with NheI and BglII and insertion of this 539 bp fragment into NheI and BglII digested pCMV6a120D3-D4-D5.

Expression vector pCMV6a can be regenerated by excising the coding sequence for gp120 from pCMV6ARV120tpa with SalI. The mutein coding sequences described above can all be constructed from the wild-type gp120 coding sequence in pCMV6ARV120tpa as described for pSV7dARV120tpa. Table 3 sets forth the names and deletions of the various M13-pSV7d- and pCMV6a-based vectors made according to the above protocol.

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30

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Table 3

	Gene Version	M13 Vector	pSV7d Vector	pCMV6a Vector
5	Wild-type	M13pSV7d120	pSV7dARV120tpa	pCMV6ARV120tpa
	D(1+2)	M13pSV7d120D1+2	not made	pCMV6a120D1+2
	D1	M13pSV7d120D3	pSV7d120D1	pCMV6a120D1
	D2	M13pSV7d120D2	pSV7d120D2	pCMV6a120D2
10	D3	M13pSV7d120D3	pSV7d120D3	pCMV6a120D3
	D4	M13pSV7d120D4	not made	pCMV6a120D4
	D6	M13pSV7d120D5	not made	pCMV6a120D5
	D3+D4	M13pSV7d120D3+D4	not made	not made
	D4+D5	M13pSV7d120D4+D	pSV7d120D4+D5	not made
15	D3+D4+D5	not made	pSV7d120D3+D4 +D5	pCMV6a120D3+D4 +D5
	D(1-5)	not made	not made	pCMV6a120D1-5
	D(1+2+5)	not made	not made	pCMV6a120D(1+2 +5)

20

pSV7dARV120tPA (Figure 3) was constructed as follows. An env gene was modified by in vitro mutagenesis to eliminate any potential transmembrane domains and to provide a stop codon following the processing site between the gp120 and gp41 domains of the gp160 protein. This mutagenesis was accomplished by subcloning the fragment which encodes the env gene from clone pSV7c/env (ATCC Accession No. 67593) by excising with HindIII and XhoI (SF2 clone positions 5582 and 8460) and inserting the 2.8 kb fragment into M13mp19 previously digested with HindIII and SalI. A 37 bp oligonucleotide of the following sequence was used to alter the sequence at the gp120/gp41 processing site at position 7306 to encode 2 stop codons and two restriction endonuclease sites.

35

5'-GAACATAGCTGTCGACAAGCTTCATCATCTTTTTTCT-3'

The sequence of the wild-type gene and the mutant are shown below:

5

Wild-type sequence:

Processing Site

Position: 7288 7294 7300 7318

Amino acid: Val Gln Arg Glu Lys Arg Ala Val Gly Ile Val

DNA: GTG CAG AGA GAA AAA AGA GCA GTG GGA ATA GTA

10

gp120 mutant:

Position: 7288 7294 7300 7306 7317

Amino Acid: Val Gln Arg Glu Lys Arg OP OP

DNA: GTG CAG AGA GAA AAA AGA TGA TGA AGC TTG TCG AC

Restriction site: HindIII SalI

15

Following mutagenesis, the gene was engineered for optimal secretion into the medium. A 268 bp XbaI-NdeI fragment containing the heterologous 5' untranslated sequences and signal sequences from human tPA fused to the 5' end of env was excised from the M13 clone M13tpaS.NheIenv described below. This fragment was ligated with a 1363 bp NdeI-SalI fragment encoding the remainder of the gp120 coding region which was isolated from the gp120 mutant (positions 5954 and 7317) described above, and both fragments were inserted into the vector pSV7d (described below) previously digested with XbaI and SalI.

30 Expression vector pSV7d can be generated by digesting pSV7c/env with BglII and XbaI, and then ligating the digested plasmid with the following linker:

BglII EcoRI SmaI XbaI BamHI SalI

5' -GATCTCGAATTCCTCCCGGGTCTAGAGGATCCGTCGAC
ABCTTAAGGGGCCAGATCTCCTAGGCACGTGGATC-5'

M13tpaS.NheIenv was constructed as follows. The 5' end of the env coding sequence was modified to accept a heterologous signal sequence known to direct efficient secretion of both the homologous gene (human tissue plasminogen activator) and deletion variants of this gene. van Zonnefeld et al., (1986) Proc. Natl. Acad. Sci. USA 83:4670.

A portion of the HIV-1 SF2 gene in a lambda phage (ATCC Accession No. 40143) was excised with SacI and StuI (positions 5555 and 6395) and was subcloned into the vector M13mp19 [Yanisch-Perron et al., (1985) Gene 33:103-109] between SacI and SmaI. Oligonucleotide-directed mutagenesis [Zoller et al., (1983) Meth. Enzymol. 100:468-500] was used to create an NheI site at the junction of the natural signal peptide and the mature envelope polypeptide using the following oligonucleotide:

5'-GATGCTCTGTTTCAGCTAGCGAAAAATTGTGG-3'

This mutagenesis changes cytosine-5867 to guanine and adenine-5868 to cytosine, thereby creating an NheI site and altering the codon for threonine-30 to code for serine.

In parallel, the tPA gene was likewise mutagenized in M13 to place an NheI site near the carboxyl end of the tPA signal peptide. The following sequences show the 5' UT sequences and signal for wild-type tPA leader and for the NheI variant.

Wild-type sequence of the tPA signal:

30

5' untranslated sequences

35

AGAGCTGACATCCTACAGGAGTCCAGGGCTGGAGAGAAAACCTCTGCGAGGAAAGGGAAGGA

GCAAGCCGTGAATTTAAGGGACGCTGTGAAGCAATCATGGATGCAATGAAGAGAGGGGCTC
MetAspAlaMetLysArgGlyLeu

5 TGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGCCAGGAAATCCATGCC
CysCysValLeuLeuLeuCysGlyAlaValPheValSerProSerGlnGluIleHisAla

mature tPA
CGATTTCAGAAGAGGAGCCAGA TCTTACCAAGTG
ArgPheArgArgGlyAlaArg SerTyrGlnVal

10

The *Nhe*I variant of the tPA signal:

5' untranslated sequences

15

AGAGCTGAGATCCTACAGGAGTCCAGGGCTGGAGAGAAAACCTCTGCGAGGAAAGGGAAGGA

GCAAGCCGTGAATTTAAGGGACGCTGTGAAGCAATCATGGATGCAATGAAGAGAGGGGCTC
MetAspAlaMetLysArgGlyLeu

20

TGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGCGCTAGC
CysCysValLeuLeuLeuCysGlyAlaValPheValSerProSerAlaSer

Following mutagenesis and sequence verification,
a 174 bp fragment containing 99 bp of 5' untranslated
sequence and the signal sequence from tPA was excised from
the tPA-containing M13 clone using *Sal*I and *Nhe*I and fused
25 to the 559 bp fragment containing the 5' end of the env
gene which was excised from the env-containing M13 clone
with *Nhe*I and *Hind*III (contributed by the M13 polylinker),
and these fragments were subcloned into M13mp18 between
*Sal*I and *Hind*III to give plasmid M13tpaS.*Nhe*Ienv. The DNA
30 and amino acid sequence of the tPA signal fused to the 5'
end of env gene is:

35

...tPA signal... 5869 (amino acid 31 of env)
Phe Val Ser Pro Ser Ala Ser Glu Lys Leu Trp Val Thr Val
TTC GTT TCG CCC AGC GCT AGC GAA AAA TTG TGG GTC ACA GTT
NheI

Example II

5 This example describes the expression of HIV-1
env analogs in mammalian cells.

DNA encoding the complete env gene with the
substituted tPA signal sequence was excised from the
plasmid pSV7dARV120tpa using the restriction endonuclease
10 SalI and inserted into the unique SalI site of the mam-
malian cell expression vector pCMV6a, and the resulting
plasmid DNA was screened to verify the correct orientation
of the gene with respect to the promoter and
polyadenylation signals (see Example I). The resulting
15 plasmid, pCMV6ARV120tpa, and the plasmid pSV7dARV120tpa
were used to transfect COS-7 cells to test expression and
secretion of gp120 into the medium. pCMV6ARV120tpa was at
least 50-fold more efficient in expressing gp120 compared
with the pSV7dARV120tpa expression plasmid in these cells.

20 Permanent cell lines were isolated as follows:
human kidney 293 cells were plated at a density of 50-70%
confluency in DME supplemented with glutamine (292 mg/L),
sodium pyruvate (110 mg/L), glucose (4.5 g/L), penicillin
(1000 U/L), streptomycin (1000 U/L), 3.7 g/L sodium bi-
25 carbonate, and fetal calf serum (10% v/v). Cells were
exposed to a calcium phosphate coprecipitate following
standard techniques with 10 ug each of the HIV env expres-
sion plasmid pCMV6ARV120tpa (wild type or deletion mutant)
and a plasmid encoding the selectable marker neomycin-
30 resistance, pSV2neo (Ref), for six hours at 37°C in a 10%
CO₂ atmosphere. Cells were washed and exposed to a 3 to 4
minute shock of 15% DMSO or glycerol in HEPES-buffered
saline, and growth medium (described above) was replaced
for 48 hours. Trypsinized cells were replated at a lower
35

density in 400 ug/ml G418 (Sigma) in DME supplemented as above. Colonies grew to the 100 cell per focus stage in one week to ten days, and these colonies were transferred individually to 96 well plates. Clones were screened for
5 gp120 production by testing the conditioned cell medium using an ELISA described below. Positive clones were scaled up to T75 flasks, aliquots of cells frozen, and cell supernatants were collected for further characterization, e.g. CD4 binding.

10 Alternatively, CHO dhfr- cells plated at a density of 50-70% confluence were cotransfected by calcium phosphate coprecipitation using 10 ug each of the plasmids pCMV6ARV120tpa (or analogous deletion mutant expression vector) and the selectable marker dhfr encoded in the
15 plasmid pAd-dhfr. Following exposure to the coprecipitate and shock solutions as described above, cells were incubated for 48 hours in Ham's F12 supplemented with glutamine (292 mg/L), sodium pyruvate (110 mg/L), sodium bicarbonate (3.7 g/L), glucose (4.5 g/L), penicillin (1000
20 U/L), streptomycin (1000 U/L), proline (150 mg/L), and fetal calf serum (10%). Forty-eight hours after transfection, cells were plated at a density of approximately one tenth in DME supplemented as described for F12 above, except that the fetal calf serum was replaced
25 with dialyzed fetal calf serum (10%). Colonies were transferred individually to 96 wells after about two weeks, then screened using the ELISA assay for gp120 secreted into the medium. Positive clones were scaled up as described above.

30 For detection of gp120 or gp120 hypervariable region deletion mutants in the supernatants of COS, CHO, or 293 cells, conditioned medium was assayed by ELISA specific for gp120 sequences. Pooled HIV-positive human serum inactivated by treatment with psoralen was affinity purified
35 on Staphylococcus Protein A Sepharose by standard

techniques. This serum was coated on Immulon 1 96 well ELISA plates at a concentration of 5 ug/ml in PBS and plates were incubated 12 hours to two months at 4°C. Following incubation, plates were washed as described for the titration ELISA (Example IV), and samples and standards (including purified recombinant HIV-1 gp120env from yeast) were applied to the plate in two-fold dilution series using the dilution buffer described for the titration ELISA. The range of the assay is 100 ng/ml to 200 pg/ml. Samples were incubated for 12 hours at 4°C. Samples were aspirated and plates were washed as above. Samples were then incubated with an appropriate dilution of rabbit serum from rabbits immunized with recombinant SF2 gp120env analog (usually 1:100 dilution of Protein A Sepharose affinity-purified serum in dilution solution) for 1 hour at 37°C, followed by washing. Color development was with ABTS, as described for the titration ELISA. Plates were read as described, and the amount of gp120 in each sample was determined by using a standard curve derived from the standard on the same plate. The assay was verified by showing that HIV-infected HUT 78 cells (infected cell lysate) gave a positive signal, while uninfected cell lysates were negative.

Example III

This example describes the recombinant production of muteins in yeast hosts according to the present invention.

The starting plasmid used for the construction of yeast expression vectors was plasmid pJS150. This plasmid is similar to plasmid pBS24.1/SOD-SF2env4-5 (U.S. serial no. 138,894, filed 24 December 1987, supra), and has had the yeast promoter and HIV coding sequences located between the unique BamHI and SalI sites replaced with a yeast ADH2/GAPDH promoter and a portion of an Zairan HIV-1 isolate envelope coding sequence. In addition, the NheI restric-

tion site located in the plasmid vector portion was destroyed by cutting with NheI, nuclease S1 treatment, followed by ligation.

Plasmid pJS150 was digested with restriction enzyme NcoI, which cuts just after the translation initiation ATG codon downstream from the ADH2/GAPDH promoter (as well as at other sites in the vector). The fragments were ligated to an NcoI/NheI adaptor having the sequence:

10 5'-CATGGCTAGCCCC
 CGATCGGGG-5'

After ligation, the DNA sequences were digested with BamHI and NheI to generate a 1.2 kb BamHI-NheI fragment containing the ADH2/GAPDH promoter and an NheI sticky end immediately downstream. This DNA fragment was isolated by gel electrophoresis and is referred to as Fragment A.

A second digest was performed by cutting plasmid pJS150 with BamHI and SalI to generate a 13 kb linear DNA vector. This DNA fragment was also purified by gel electrophoresis, and is referred to as Fragment B. A third DNA fragment containing a coding sequence for gp120 D3 mutein was isolated from pCMV6a120D3 (Table 3 of Example I) by digestion with NheI and SalI. The approximate 1.2 kb coding fragment was then gel isolated, and is referred to as Fragment C.

The three fragments (A, B and C) were then ligated together, and the resulting ligation mix was used to transform E. coli strain HB101 to ampicillin resistance. A plasmid containing all three fragments in the proper orientation is shown in Figure 5. This D3 yeast expression vector was designated pHL15. Expression vectors for additional muteins were also constructed by cloning the mutein-encoding sequence of the pCMV6a-based vectors (Example I, Table 3) into NheI/SalI-digested pHL15, thereby

replacing the D3 coding sequence. The deletions and corresponding vectors that were made are shown in Table 4.

Table 4

5		<u>Deletion</u>	<u>Vector</u>
		D1	pHL24
		D2	pHL25
10		D3	pHL15
		D4	pHL26
		D5	pHL27
		D1 + D2	pHL22
		D3 + D4 + D5	pHL21
15		D1 through D5	pHL20

The yeast expression vectors described above were used to transform Saccharomyces cerevisiae strain JSC308 (ATCC accession No. 20879, deposited 5 May 1988) to uracil prototrophy. Uracil prototrophs were then streaked onto leucine selective plates to isolate leucine prototrophs (as a result of plasmid amplification in vivo). Expression of the deletion muteins was achieved by growing a seed culture of the leucine prototrophs in leucine selective medium and then diluting it into approximately 10 liters of a rich medium containing yeast extract, peptone, and glucose. Either 2% or 4% glucose was used as the carbon source in the media, whichever appeared optimal.

Muteins were purified as follows. Frozen yeast cells were thawed and suspended in 1 volume of lysis buffer, 0.001 M PMSF, 0.001 M EDTA, 0.15 M NaCl, 0.05 M Tris-HCl pH 8.0), and 1 volume of acid-washed glass beads added. Cells were broken in a noncontinuous system using a 300 ml glass unit of Dyno-mill at 3000 rpm for 10 min. the jacket was kept cool by a -20°C ethylene glycol solution.

Glass beads are decanted by letting the mixture set for 3 min on ice. The cell extract was recovered and centrifuged at 18,000 rpm (39,200 x g) for 35 min. The supernatant was discarded and the precipitate (pellet 1) further treated as indicated below.

Pellet 1 was resuspended in 4 volumes of Tris-HCl buffer (0.01 M Tris-HCl, pH 8.0, 0.01 M NaCl, 0.001 M PMSF, 1 ug/ml pepstatin, 0.001 M EDTA, 0.1% SDS) and extracted for 1 hr at 4°C with agitation. The solution was centrifuged at 6,300 x g for 15 min. The insoluble fraction (pellet 2) was resuspended in 4 volumes (360 ml) of PBS (per liter: 0.2 g KCl, 0.2 g KH₂PO₄, 8.0 g NaCl, 2.9 g Na₂HPO₄·12H₂O), 0.1% SDS, 0.001 M EDTA, 0.001 M PMSF, 1 ug/ml pepstatin, and centrifuged at 6,300 x g for 15 min. This pellet (pellet 3), was suspended in 4 volumes of PBS, 0.2% SDS, 0.001 M EDTA, 0.001 M PMSF, 1 ug/ml pepstatin and extracted for 12 hr at 4°C with agitation on a tube rocker. The solution was then centrifuged at 6,300 x g for 15 min. The soluble fraction was recovered for further purification as indicated below. (The pellet can be reextracted by resuspending it in 4 volumes of 2.3% SDS, 5% beta-mercaptoethanol, and boiling for 5 min. After boiling, the solution is centrifuged at 6,300 x g for 15 min. The soluble fraction is recovered for further purification.)

The soluble fraction was concentrated by precipitation with 30% ammonium sulfate at 4°C. The pellet (pellet 4) was then resuspended in 2.3% SDS, 5% beta-mercaptoethanol, and chromatographed on an ACA 34 or ACA 54 (LKB Products) gel filtration column (depending on the size of the mutein). The column was equilibrated with PBS, 0.1% SDS, at room temperature. Chromatography was developed in the same solution with a flow rate of 0.3 ml/min. If needed, pooled fractions were concentrated by vacuum dialysis on Spectrapor #2 (MW cutoff 12-14K).

Example IV

This example describes an immunoassay for anti-HIV antibodies employing HIV-1 env analogs.

Immulon-1 96 well immunoassay plates are coated
5 with gp120 antigen by dispensing 100 ul per well of a 2 ug
per ml purified antigen produced in yeast (Env SF2 wild
type, env HTLV wild type, env Zr6 wild type, env SF2-D3,
env SF2-D1-D2, env SF2-D3-D4-D5, env SF2-D1-D2-D3-D4-D5 in
50 mM borate pH 9.2 at 4°C for at least 12 hours and less
10 than 60 days. The coating is aspirated from the plate, and
the plate is washed six times by dunking in a solution of
0.137 M NaCl (0.8%), 0.05% Triton-X 100. The plate is pat-
ted dry, and 100 ul per well of 100 mM sodium phosphate,
0.1% casein, 1 mM EDTA, 1% Triton-X 100, 0.5 M NaCl, 0.01%
15 thimerosal pH 7.5 (dilution solution) is added.

Sera to be tested are prepared for analysis by
diluting 5 ul test serum (from HIV-positive humans and
normal humans, or from immunized animals) in 500 ul of the
dilution solution above (1/100 dilution v/v). The solution
20 in the top wells is aspirated off and 150 ul of diluted
test serum is added. Using a multichannel pipettor set at
50 ul, dilutions down each column are carried out, taking
50 ul each time (1/3 dilution v/v). The plates are then
incubated 1 hour at 37°C with the plate wrapped in plastic
25 wrap. The samples are then aspirated off and washed 6
times as above. Then, 100 ul per well of goat anti-human
IgG conjugated to horseradish peroxidase (Tago 2733 Lot
330102) diluted 1/2000 in dilution solution is added. The
plates are then incubated 30 minutes at 37°C covered in
30 plastic wrap. The solution is again aspirated off and
washed 6 times as above. 100 ul per well of color develop-
ing solution is added [100 ul ABTS stock (15 mg/ml 2,2'-A
zino-di-(3 ethylbenzthiazolene sulfonic acid), Sigma A-
1888, in water, stored in the dark at 4°C) plus 3.3 ul 30%
35 hydrogen peroxide in 10 ml citrate buffer (10.5 g citric

acid per liter water, pH to 4.0 with 6M NaOH)]. The ABTS solution is made no more than 10 minutes prior to use, and the solution should be made with citrate buffer at room temperature. Plates in plastic wrap are incubated at 37°
5 for 30 minutes in the dark. The reaction was stopped with 50 ul per well of 10% sodium dodecyl sulfate.

Plates were read at 415 nm with a reference wavelength at 600 nm. Titters are determined as follows from the raw data: absorbance (linear axis) is plotted vs.
10 dilution factor (log axis) on semilog paper or computer program.

Included in the test sera are standard reference sera as positive controls. For titration of human sera, HIV-positive serum 20058 from the Interstate panel was
15 used; for goat sera, a goat serum developed by immunization with envSF2 wild-type, reference 02GT097.2 was used; and for titration of guinea pig sera, a standard reference guinea pig serum was used, likewise obtained by immuniza-
20 tion with envSF2 wild-type, reference +Gp sera 1935/77. From the values obtained on the ELISA reader, an absorbance value was chosen that is half-maximal (OD₅₀) (between 0.5 and 0.7) in the linear portion of the standard curve (positive control), for each plate. The average titer for the standards on the plate were determined. The average
25 titer was divided by the "Reference Titer" for that species and antigen plate, and the resulting number is the "correction factor". The test sera titers were divided by the correction factor to yield the adjusted, normalized titers.

A panel of selected human sera was tested to
30 determine their titers on all of the recombinant antigens, including the deletion muteins env SF2 D1-2, env SF2-D-3, env SF2-D1-2-3-4-5, and env SF2-D3-4-5. Results are shown in Figure 6 (North American serum panel) and Figure 7 (African serum panel). The graphs show that for these
35 serum samples, the recombinant antigen env SF2-D1-2 is as

efficient or more efficient in detecting sera as is env SF2 wild-type. Recombinant antigens env SF2-D3, env SF2-D3-4-5 and env SF2-D1-2-3-4-5 are as efficient as env HTLVIII wild-type or env Zr6 wild-type.

5

Example V

This example describes the immunization of mammals with recombinant hypervariable region muteins and the
10 detection of immune responses in these animals in response to these injections. Recombinant env antigens purified from yeast were used to generate anti-HIV antibodies in experimental animals of very high titer. In both goats and guinea pigs, the titers of the resulting sera were at a
15 similar level, whether the immunogen was env SF2 wild-type or an env SF2 hypervariable mutein.

Immunization of Guinea Pigs

In order to test if guinea pigs immunized with env
20 SF2 muteins derived from yeast were capable of generating a strong immune response, these animals were immunized with several muteins and control antigens.

For each antigen, six Hartley guinea pigs were immunized in the footpad with 50 ug each antigen mixed with
25 50 ug adjuvant (see below) at three week intervals with a total of seven injections. Blood samples were taken at the time of each injection (prebleed at injection 1), and the serum was monitored by titration in the ELISA described above in Example IV for the production of antibodies
30 directed against the immunizing antigen and against heterologous env antigens.

35

-39-

Antigens:

- Env SF2-D3
- Env SF2-D-1-2-3-4-5
- Env SF2-D3-4-5
- 5 Env HTLVIII wild-type

Adjuvant:

Muramyl-tripeptide-phosphatidyl ethanolamine in squalene-Tween.

10

Vaccine:

- Mix adjuvant with correct volume of 10X Squalene-Tween (carrier) to make a final concentration of 1X (4% squalene, 0.008% Tween-20), antigen, and PBS to make the correct volume and dose (50 ug each of antigen and adjuvant per animal in 100 ul injection volume per animal. Warm the mixture for 5 min at 45°C. Pass the warm mixture in and out of a 23 ga. needle six times, taking care to avoid introducing air into the mixture. Inject into the animals immediately. If the injection procedure takes more than 5 min, remix the emulsion by shaking by hand every few minutes.

Control:

- 25 Control animals receive the adjuvant in carrier.

Results:

- The results of the ELISA are shown in Figure 8. As can be seen, the muteins of the present invention are as effective as wild-type envelope in generating high antibody titers. A standard virus neutralization assay was also conducted the guinea pig sera. The results are shown in Table 5. The data show that deletion mutants of the present invention can generate significant levels of neutralizing antibodies.

35

Table 5

<u>Virus Neutralization by Guinea Pig Sera</u>			
5	Antigen	Animal Number	ELISA Titer/ env SF2 Neutralization Titer HIV-SF2
10	Env SF2 D3	2477	280,000 50
	(COOH half)	2476	221,000 500
		2479	274,000 250
		2480	190,000 100
		2481	115,000 <20
15	Env SF2 D3	2471	45,000 >500
		2473	57,000 <20
		2474	116,000 <20
		2475	26,000 <20
		2476	16,000 <500
20	Env SF2 D(3-5)	2489	342,000 500
	(full length)	2490	88,000 30
		2491	131,000 45
		2492	83,000 25
		2494	253,000 30
25	Env SF2 D(1-5)	2483	131,000 <20
		2484	71,000 <20
		2485	60,000 <20
		2486	54,000 <20
		2487	100,000 <20
30		2488	133,000 <20
35	Env HTLVIII	2523	23,000 <20
		2524	37,000 <20
		2525	52,000 <20
		2526	23,000 <20
		2527	70,000 <20
		2528	14,000 <20

Immunization of Goats

In order to test if goats immunized with env SF2 muteins derived from yeast were capable of generating a strong immune response, these animals were immunized with several muteins and control antigens.

For each antigen, two goats were immunized intramuscularly with 1 mg each primary injection (complete Freund's adjuvant) and 0.5 mg each booster injection (incomplete Freund's adjuvant) at three week intervals with a total of six injections. Blood samples were taken at the time of each injection (prebleed at injection 1), and the sera were monitored by titration in the ELISA assay described above in Example IV for the production of antibodies directed against the immunizing antigen and against heterologous env antigens.

Antigens:	Env SF2-D3	Lot 3064/1a
	Env SF2-D1-a2-3-4-5	Lot 3064/5a
	Env HTLVIII wild-type	Lot 3064/15a

Adjuvant: Complete Freund's and incomplete Freund's.

Vaccine: Mix 0.5 ml antigen (1 mg) with 0.5 ml complete Freund's. Emulsify by standard procedures and inject. For boosters, mix 0.5 ml antigen (0.5 mg) with 0.5 ml incomplete Freund's. Emulsify and inject.

Control: Control animals receive the adjuvant in carrier.

Results: The results of the ELISA are shown in Figure 9. As can be seen, the muteins of the present invention are at least as effective as wild-type polypeptides in antibody titer levels generated.

Deposit of Biological Materials

Vectors pCMV6ARV120tpa and pHL15, both in E. coli HB101, were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA, on 13 September 1988, and will be maintained under the provisions of the Budapest Treaty on the Deposit of Microorganisms. The accession number for the pCMV6ARV120tpa deposit is 67792, and the accession number for the pHL15 deposit is 67793.

These deposits are provided for the convenience of those skilled in the art, and are neither an admission that such deposits are required to practice the present invention, nor that equivalent embodiments are beyond the skill of the art in view of the present disclosure. The availability of these deposits is not a grant of any license (e.g., to make, use or sell the deposited materials) under this or any other patent. The nucleic acid sequences of the deposited materials are incorporated in the present disclosure by reference and are controlling if in conflict with any sequence described herein.

Although the foregoing invention has been described in some detail by way of illustration and example, it will be obvious that changes and modifications may be practiced within the scope of the appended claims.

30

35

CLAIMS

1. An improved polypeptide analog of human
5 immunodeficiency virus type 1 (HIV-1) gp120env or
gp160env, the improvement comprising the deletion of at
least one epitope in a hypervariable domain, while retain-
ing the domains conserved among HIV-1 isolates.

10 2. A polypeptide comprising epitopes bound by
antibodies to HIV-1 gp120env or gp160env and an amino acid
sequence according to the formula:

C1-V1-V2-C2-V3-C3-V4-C4-V5-C5

15 wherein:

C1 is an amino acid sequence substantially
homologous to Ser29 through Cys130 of HIV-1 SF2;

20 C2 is an amino acid sequence substantially
homologous to Cys199 through Leu291 of HIV-1 SF2;

C3 is an amino acid sequence substantially
homologous to Ser366 through Cys387 of HIV-1 SF2;

C4 is an amino acid sequence substantially
homologous to Cys415 through Gly456 of HIV-1 SF2;

25 C5 comprises an amino acid sequence
substantially homologous to Phe466 through Arg509 or
Leu855 of HIV-1 SF2;

V1 is an amino acid sequence of 0 to a maximum
of about 30 residues;

30 V2 is an amino acid sequence of 0 to a maximum
of about 50 residues;

V3 is an amino acid sequence of 0 to a maximum
of about 90 residues;

35 V4 is an amino acid sequence of 0 to a maximum
of about 30 residues; and

V5 is an amino acid sequence of 0 to a maximum of about 10 residues;

with the proviso that at least one of the V domains selected from the group consisting of V1, V2, V3, V4 and V5 contains no more than about one-third of the said maximum number of residues for the V domain.

3. The polypeptide of claim 2 wherein:

V1, if present, comprises an HIV-1 sequence spanning from the C-termini of C1 to a Cys corresponding to the conserved Cys155 of HIV-1 SF2;

V2, if present, comprises an HIV-1 sequence spanning from said C-termini Cys of V1 to the N-termini of C2;

V3, if present, comprises an HIV-1 sequence spanning from the C-termini of C2 to the N-termini of C3;

V4, if present, comprises an HIV-1 sequence spanning from the C-termini of C3 to the N-termini of C4; and

V5, if present, comprises an HIV-1 sequence spanning from the C-termini of C4 to the N-termini of C5;

with the proviso that for said selected V domain having no more than one-third the maximum residue, if said HIV-1 sequence is present, said HIV-1 sequence is missing an epitope.

4. The polypeptide of claim 2 wherein said selected V domain is V1.

5. The polypeptide of claim 2 wherein said selected V domain is V2.

6. The polypeptide of claim 2 wherein said selected V domain is V3.

7. The polypeptide of claim 2 wherein said selected V domain is V4.

8. The polypeptide of claim 2 wherein said
5 selected V domain is V5.

9. The polypeptide of claim 2 wherein said selected V domains are V1 and V2.

10. The polypeptide of claim 2 wherein said
10 selected V domains are V1, V2 and V3.

11. The polypeptide of claim 2 wherein said selected V domains are V1, V2 and V5.

12. The polypeptide of claim 2 wherein said
15 selected V domains are V3, V4 and V5.

13. The polypeptide of claim 2 wherein said
20 selected V domains are V1, V2, V3, V4 and V5.

14. A polypeptide analog of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env comprising (a) about 300 to about 850 amino acid residues
25 in length; (b) constant domains, in the N-termini to the C-termini direction, Ser29-Cys130 of HIV-1 SF2 (C1), Cys199-Leu291 of HIV-1 SF2 (C2), Ser366-Cys387 (C3), Cys415-Gly456 (C4) of HIV-1 SF2, and Phe466-Arg509 or Phe466-Leu855 of HIV-1 SF2 (C5), or domains substantially
30 homologous to said C1, C2, C3, C4 or C5; and (c) the intervening domains, if any, located between said constant domains comprising sequences found between said constant domains in native HIV-1 gp120env, with the proviso that at least one of said intervening domains between said
35 constant domains is either missing or missing an epitope.

15. The polypeptide of claim 14 wherein said missing epitope is a neutralizing epitope.

16. The polypeptide of claim 14 wherein said
5 intervening domains are HIV-1 SF2 sequences.

17. The polypeptide of claim 16 comprising an HIV-1 SF2 gp120env deletion mutein wherein C5 is Phe466-Arg509 and at least one deletion is selected from the
10 group consisting of:

- (a) Thr131-Asn154;
 - (b) Ser156-Ser198;
 - (c) Thr300-His332;
 - (d) Asn388-Pro414; and
 - 15 (e) Thr457-Thr463;
- or a polypeptide substantially homologous thereto.

18. The polypeptide of claim 17 wherein said deletion comprises Thr131-Asn154.
20

19. The polypeptide of claim 17 wherein said deletion comprises Ser156-His198.

20. The polypeptide of claim 17 wherein said
25 deletion comprises Thr300-His332.

21. The polypeptide of claim 17 wherein said deletion comprises Asn388-Pro414.

22. The polypeptide of claim 17 wherein said
30 deletion comprises Thr457-Thr463.

23. The polypeptide of claim 17 wherein said deletions comprise Thr131-Asn154 and Ser156-His198.
35

24. The polypeptide of claim 17 wherein said deletions comprise Thr131-Asn154, Ser156-His198 and Thr457-Thr463.

5 25. The polypeptide of claim 17 wherein said deletions comprise Thr300-His332, Asn388-Pro414 and Thr457-Thr463.

10 26. The polypeptide of claim 17 wherein said deletions comprise Thr131-Asn154, Ser156-His198, Thr300-His332, Asn388-Pro414 and Thr457-Thr463.

15 27. An immunoassay for the detection of antibodies to human immunodeficiency virus type 1 (HIV-1) comprising:

(a) providing a liquid sample to be tested for the presence of anti-HIV-1 antibodies;

(b) contacting said sample with a polypeptide according to claim 1 under conditions whereby any anti-HIV-1 antibodies present in said sample may bind to an epitope to provide sample-contacted polypeptide; and

(c) detecting any antibody bound to said sample-contacted polypeptide.

25 28. A method of selectively raising antibodies in a mammal to epitopes in the constant domains of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env comprising administering to said mammal a polypeptide of claim 1, whereby antibodies to said polypeptide are produced in response to said administration.

30 29. A composition comprising a polypeptide according to claim 1 and a pharmaceutically acceptable carrier.

30. The composition of claim 29 further comprising an adjuvant.

31. A DNA sequence encoding a polypeptide according to claim 1.

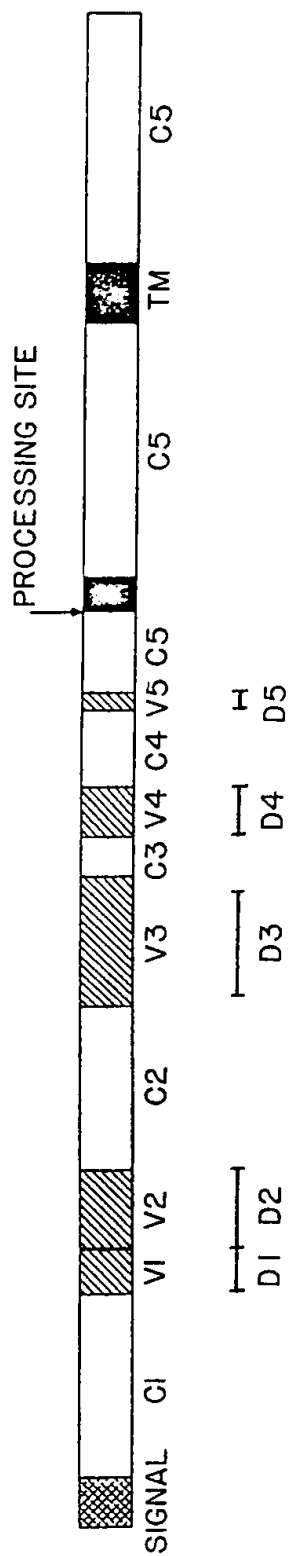
32. A cellular host comprising the DNA sequence of claim 31 under the control of transcriptional and translational control sequences whereby the polypeptide encoded by said DNA sequence is expressed by said cellular host.

33. The cellular host of claim 32 wherein said cellular host is a mammalian cell.

34. The cellular host of claim 32 wherein said cellular host is a yeast cell.

35. A method of producing a recombinant analog of human immunodeficiency virus type 1 (HIV-1) envelope protein comprising growing culture of cellular hosts according to claim 32 under conditions whereby said polypeptide encoded by said DNA sequence is expressed, and recovering said polypeptide from said culture.

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HIV-SF2env
2565bp

FIG. 1

SUBSTITUTE SHEET

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HXB2	MetArgValLysGluLys.....TyrGlnHisLeuTrpArgTrpGlyTrpArgTrp	17
BRU	-----Lys-----	17
MN	-----GlyIleArgArgAsn-----	16
SC	-----GlySerGlyArgAsn-----	16
SF2	---Lys---GlyThrArgArgAsn-----	16
NY5	---Ala---GlyThrArgLysAsn-----	16
CDC4	---Ala---GlyIleArgLysAsnCys-----	16
WMJ2	---GlyIleMetArgAsnCys-----Ile-----	16
RF	---Met---MetArgLysAsnCys-----Lys-----	16
MAL	---Arg---IleGlnArgAsn-----AsnTrp-----	16
ELI	---AlaArgGlyIleGluArgAsnCys-----AsnTrp---Lys-----	16
Z6	---AlaArg---IleGluArgAsnCysProAsn-----Lys-----	16
Z3	---IleGlnArgAsn-----Lys-----	16
Z321	---Lys---GlyIleGlnGlyAsnTrp---AsnTrp---Lys-----	16
JY1	---MetGlyIleArgMetAsn-----Lys-----	16

SUBSTITUTE SHEET

FIG. 2-1A

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signal peptide \ / gp120		* → CL	
HXB2	GlyThrMetLeuLeuGlyMetLeuMetIleCysSerAlaThrGluLysLeuTrpValThr		37
BRU	Ile		37
MN	Leu		36
SC	Ile	Ala	36
SF2	Leu	Gln	36
NY5		Ala	36
CDC4		AlaAlaAsn	36
WMJ2	Phe	Val	36
RF	Trp	Gln	36
MAL		Ala	36
ELI	Met	Asp	36
Z6	Ile	IleAla	36
Z3	Ile	Asp	36
Z321	SerLeuIleIle	AlaAspAsn	36
JY1	LeuIle	Lys	36
	Ile	Ile	36
	LeuValIle	Ala	36
	Ile	Asn	36
	Thr	ValAla	36
		Asp	36

FIG. 2-1B

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HXB2	ValTyrTyrGlyValProValTrpLysGluAlaThrThrThrLeuPheCysAlaSerAsp	57
BRU	-----	57
MN	-----	56
SC	-----	56
SF2	-----	56
NY5	-----	56
CDC4	-----	56
WMJ2	-----	56
RF	-----Glu	56
MAL	-----	56
ELI	-----	56
Z6	-----	56
Z3	-----Asp-----Glu	56
Z321	-----Asp-----Glu	56
JY1	-----	56

SUBSTITUTE SHEET

FIG. 2-2A

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HXB2	AlaLysAlaTyrAspThrGluValHisAsnValTrpAlaThrHisAlaCysValProThr	77
BRU	-----	77
MN	-----	76
SC	-----Gln-----	76
SF2	-----Ile-----	76
NY5	---Arg-----	76
CDC4	-----	76
WMJ2	-----Ala-----	76
RF	---Ser---Ala---	76
MAL	---Lys---Lys---	76
ELI	---Ser---Glu---Ile---	76
Z6	---Ser---Glu---Ala---Ile---	76
Z3	---Ser---Lys---Ala---Ile---	76
Z321	---GluLys---Ser---	76
JY1	---Lys---	76
	---Ser---GluPro---Ala---Ile---	76

FIG. 2-2B

SUBSTITUTE SHEET

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HXB2	AsnAspMetValGluGlnMethHisGluAspIleIleSerLeuTrpAspGlnSerLeuLys	117
BRU	-----	117
MN	---Asn-----	116
SC	---Asn-----	116
SF2	---Asn-----Gln-----	116
NY5	---AsnThr-----	116
CDC4	---Asn-----	116
WMJ2	---Asn-----	116
RF	---Asn-----	116
MAL	---Asn-----	116
ELI	---Asn-----	116
Z6	---Asn-----Ile-----	116
Z3	---Lys-----	116
Z321	---Asn-----Val-----	116
JY1	---Asn-----Asn-----	116

SUBSTITUTE SHEET

FIG. 2-3A

HXB2	[[]]	
BRUAsnAspThr...	AsnThrAsnSerSer.....Ser	144	
MN	-----Ala-----	-----AsnThrAsnSerSer-----	-----Ser	149	
SC	-----Thr-----	-----Asn-----ThrAlaAsnAsn-----	-----Asn	148	
SF2	-----...Ala-----	-----Ser-----AlaThr-----	-----AsnThrThrSerSerAsn	148	
NY5	-----Ala-----TyrAla...	-----Gly-----	-----AsnTrpLys-----	145	
CDC4	-----Asn-----...	-----ThrThrGlulLeuSerIleIleValVal...	-----Val	142	
WMJ2	-----Ile-----AspTrpLys-----	-----ThrThr-----IleIle-----	-----IleIle-----	149	
RF	-----Gly-----...	-----ValThr-----	-----SerSer-----	144	
MAL	-----Gly-----AlaVal...	-----GlyThrAsnAlaGlySerAsnArgThrAsn	-----Arg	144	
ELI	-----Asn-----Gly-----MetGlyAsn-----	-----ValThr-----	-----Val	149	
Z6	TrpMetGly-----Val-----GlyLys...	-----ValThr-----	-----Val	143	
Z3	-----Ser-----...	-----AsnThr-----	-----Asn	147	
Z321	Asp-----Asn-----...	-----ValAspThr-----	-----Val	141	
JY1	-----Lys-----Thr-----Gly-----	-----AsnThr-----	-----ThrAsnGln	144	
				147	

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FIG. 2-3C [*] []

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HXB2	SerIleArgGlyLysValGlnLysGluTyrAlaPhePheTyrLysLeuAspIleIlePro	183
BRU	-----	188
MN	-----Asp-----Met-----LeuLeu-----ValSer	188
SC	-----Ser-----Leu-----ValVal-----	186
SF2	-----Asp-----Ile-----Asn-----Leu-----ArgAsn-----ValVal-----	181
NY5	Ile-----Asn-----Ile-----Leu-----Arg-----Val-----	178
CDC4	-----Asp-----Arg-----Leu-----ValGlu-----	187
WMJ2	-----Arg-----Asp-----His-----Leu-----ValVal-----	178
RF	-----Arg-----Asp-----Thr-----Lys-----Leu-----ValVal-----	183
MAL	ValGlySerAsp-----Arg-----...-----Thr-----Asn-----LeuValGln	187
ELI	ValLeuLysAsp-----Lys-----GlnVal-----Leu-----Arg-----Val-----	180
Z6	ValVal-----Asp-----ThrLysGlnValHis-----Leu-----Arg-----Val-----	181
Z3	GluLeuLysAsp-----ThrGluThrValHisThrLeu-----ValVal-----	175
Z321	GluLeu-----Asp-----GlnArg-----Ile-----SerLeu-----Arg-----Val-----	179
JY1	Val-----SerAsp-----LysLysGlnValHis-----Leu-----Arg-----ValVal-----	186

FIG. 2-4B

SUBSTITUTE SHEET

HXB2	Ile.....	[.....]	"peptide T" region	189
BRU	AspAsnAspThr.....	194
MN	194
SC	190
SF2	192
NY5	184
CDC4	198
WMJ2	186
RF	199
MAL	194
ELI	190
Z6	191
Z3	186
Z321	190
JY1	201

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FIG. 2-4C

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HXB2	SerTyrSer.....LeuThrSerCysAsnThrSerValIleThrGlnAlaCysPro	206
BRU	---Thr---	211
MN	---Arg---Ile---	211
SC	---Thr---IleAsn---	207
SF2	Asn---Arg---IleHis---Arg---	209
NY5	---Thr---IleAsn---	201
CDC4	Lys---Arg---IleAsn---	215
WMJ2	Arg---Arg---IleAsn---	203
RF	---GlyAsnTyrThr---IleHis---Ser---	219
MAL	---Arg---IleAsn---	211
ELI	Asn---Arg---IleAsn---Ala---	207
Z6	Asn---Arg---IleAsn---Ala---	208
Z3	Thr---Arg---IleAsn---Thr---	203
Z321	Lys---Arg---IleAsn---Ala---	207
JY1	Asn---Arg---IleAsn---Ala---	218

D2 ← → C2
* [] *

FIG. 2-5A

SUBSTITUTE SHEET

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HXB2	LysValSerPheGluProIleProIleHisTyrCysAlaProAlaGlyPheAlaIleLeu	226
BRU	-----	231
MN	---Ile---	231
SC	-----ArgTrp....	226
SF2	-----Thr-----	229
NY5	-----	221
CDC4	-----Thr-----Thr-----Leu-----	235
WMJ2	-----	223
RF	-----Thr-----	239
MAL	---Thr---Asp---	231
ELI	-----	227
Z6	-----	228
Z3	-----	223
Z321	-----	227
JY1	---Thr-----	238

*

SUBSTITUTE SHEET

FIG. 2-5B

	* []		
HXB2	CysTnrHisGlyIleArgProValSerThrGlnLeuLeuAsnGlySerLeuAla	266	16 / 59
BRU	-----	271	
MN	-----	271	
SC	-----His-----	266	
SF2	-----Ile-----	269	
NY5	-----Lys-----	261	
CDC4	-----	275	
WMJ2	-----	263	
RF	-----	279	
MAL	-----Lys-----	271	
ELI	-----	267	
Z6	-----	268	
Z3	-----Ser-----	263	
Z3Z1	-----	267	
JY1	-----	278	

SUBSTITUTE SHEET

FIG. 2-6A

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HXB2	GluGluGluValValIleArgSerValAsnPheThrAspAsnAlaLysThrIleIleVal	286
BRU	-----Ala-----	291
MN	-----Glu-----	291
SC	-----Leu-----Glu-----	286
SF2	-----Asp-----Asn-----	289
NY5	---Gly---Glu---Asn---	281
CDC4	-----Glu-----Asn-----	295
WMJ2	-----Ile-----Glu-----	283
RF	-----Glu-----Val-----	299
MAL	-----IleMet-----Glu---Leu---Thr---Asn---	291
ELI	-----Ile-----Glu---Leu---Asn---Asn---Ala	287
Z6	-----IleIle-----Glu---Leu---Asn---Ile---	288
Z3	-----Ile-----Glu---Ile---Asn---	283
Z321	---Gly---Arg-----Glu-----Ile---	287
JY1	-----IleIle-----Glu---Leu---Asn---Val---	298

FIG. 2-6B

SUBSTITUTE SHEET

	C2	[]	[*]	D3	
HXB2	GlnLeu	Asn	Thr	Ser	Val	Glu	Ile	Asn
BRU								Asn...ThrArgLys
MN	His					Gln		
SC								Tyr---Lys---
SF2								ThrArg
NY5								
CDC4								Lys---
WMJ2	His							His---
RF								Tyr---Val---Arg
MAL								
ELI	His							Gly---Arg
Z6								Gln
Z3								Gln
Z321								GlySerAsp---LysLysIle
JY1	His							Met---
								Asp---LysIle---Gln

SUBSTITUTE SHEET

FIG. 2-6C

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FIG. 2-7A

HXB2	Arg.....IleArgIleGlnArgGlyProGlyArgAlaPheValThrIleGlyLys	322
BRU	Ser-----	327
MN	-----His-----Tyr-----ThrLysAsn	325
SC	Ser-----His-----TyrAlaThr-----Asp	328
SF2	Ser-----Tyr-----His-----Thr-----Arg	323
NY5	GLY-----Ala-----ThrLeuTyrAlaArgGlu---	315
CDC4	---ValThrLeu.....ValTrpTyr---Thr---Glu	329
WMJ2	Ser-----LeuSer-----Arg---Arg...Glu	316
RF	Ser-----ThrLys.....ValIleTyrAlaThr---Gln	333
MAL	GLY-----HisPhe.....Gln---LeuTyr---Thr-----	324
ELI	-----ThrPro-----Leu---GlnSerLeuTyr---ThrArgSer	321
Z6	Ser-----ThrPro-----Leu---Gln---LeuTyr---ThrArgGly	322
Z3	-----GlnSer---ArgIle-----LysVal---TyrAlaLys---Gly	319
Z321	Ser-----Ser-----PheAlaThr---Asp	321
JY1	Ser-----ThrPro-----Leu---Gln---LeuTyr---ThrArg...	332

SUBSTITUTE SHEET

	D3	* []	
HXB2IleGlyAsnmetArgGlnAlaHisCysAsnIleSerArgAlaLysTrp		338
BRU	-----	-----	343
MN	Ile-----ThrIle-----	-----	342
SC	Ile-----AspIle-----	-----	337
SF2	Ile-----AspIle-----Lys-----Gln-----	-----	340
NY5	Ile-----AspIle-----Lys-----	-----	332
CDC4	IleLeu-----Ile-----Gln-----	-----	346
WMJ2	Ile-----IleIle-----	-----	333
RF	Ile-----AspIle-----Lys-----Leu-----Gln-----	-----	350
MAL	IleVal-----AspIle-----Arg-----Tyr-----Thr-----AsnGluThrGlu-----	-----	341
ELI	Arg---SerIle-----.....	-----	337
Z6	ArgThrLysIle-----.....	-----	339
Z3	IleThr-----.....	-----	333
Z321	Ile-----AspIle-----Val-----ThrGlu-----	-----	338
JY1	IleLys-----AspIle-----Tyr-----Ala-----Ala-----	-----	349

SUBSTITUTE SHEET

FIG. 2-7B

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HXB2	[AsnAsnThrLeuLysGlnIleAspSerLysLeuArgGluGlnPheGlyAsnAsn...	Lys	357
BRU	---	Ala---	---	362
MN	---	Asp---	Arg---	360
SC	---	Val---	ValIle---	355
SF2	---	Glu---	ValLys---	359
NY5	---	Asp---	ValThr---	350
CDC4	---	Gln---	AlaThrThr---	364
WMJ2	---	---	ValGlu---	351
RF	---	---	ValValThr---	368
MAL	---	AspLys---	Gln---	359
ELI	---	SerLys---	Gln---	355
Z6	---	Lys---	GlnArgValAlaIle---	357
Z3	---	Arg---	Gln---	351
Z321	---	Asp---	SerLysValAlaAlaGln---	358
JY1	---	Lys---	Gln---	367

FIG. 2-7C

SUBSTITUTE SHEET

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HXB2	Thr	Ile	Ile	Phe	Lys	Gln	Ser	Ser	Gly	Gly	Asp	Pro	Glu	Ile	Val	Thr	His	Ser	Phe	Asn	377
BRU	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	382
MN	---	Val	---	Asn	---	---	---	---	---	---	---	---	---	---	Met	---	---	---	---	---	380
SC	---	---	---	Asn	Arg	---	---	---	---	---	---	---	---	---	Met	---	---	---	---	---	375
SF2	---	Val	---	Asn	---	---	---	---	---	---	---	---	---	---	Met	---	---	---	---	---	379
NY5	---	Val	---	Asn	---	---	---	---	---	---	---	---	---	---	Met	---	---	---	---	---	370
CDC4	---	Ala	---	Asn	---	---	---	---	---	---	---	---	---	---	Met	---	---	---	---	---	384
WMJ2	---	Val	---	Asn	His	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	371
RF	---	Val	---	Thr	Ser	---	---	---	---	---	---	---	---	---	Leu	---	---	---	---	---	388
MAL	Lys	---	---	Asn	Ser	---	---	---	---	---	---	---	---	Thr	---	---	---	---	---	---	379
ELI	Ile	---	Lys	---	Pro	---	---	---	---	---	---	---	---	Thr	---	---	---	---	---	---	375
Z6	---	---	---	Pro	---	---	---	---	---	---	---	---	Ala	---	Thr	---	---	---	---	---	377
Z3	Ser	---	---	Asn	Ser	---	---	---	---	---	---	Ile	---	Thr	---	Thr	---	---	---	---	371
Z321	Asp	---	---	Ala	Asn	---	---	---	---	---	---	Val	---	Thr	---	---	---	---	---	---	378
JY1	---	---	---	Pro	Pro	Ala	---	---	---	---	---	---	---	Thr	---	---	---	---	---	---	387

↑ C3

SUBSTITUTE SHEET

FIG. 2-8A

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FIG. 2-8B

	[]	
HXB2Phe	AsnSerThr...	TrpSerThr.....GluGlySerAsnAsnThrGlu
BRU	-----	-----	-----
MN	-----Asn	Gly-----	AsnAsnThrThr-----Asn...
SC	-----Gly	-----	-----Gly
SF2	-----Arg	Leu-----His-----	-----Lys
NY5	-----Leu-----	AsnAsp-----Thr-----Arg-----	-----Asp
CDC4	ValThrSer-----Gly-----	-----	-----ValThrArgLys
WMJ2	-----Gly-----	Asp-----	IleLys-----Asp-----LysAsn...
RF	-----	-----	-----Gly
MAL	-----Gln	Asn-----Gly	AlaArg.....Leu-----Ser-----
ELI	-----Ile	SerAla-----Asn	AsnIleThr-----Glu-----SerThr
Z6	-----Asn	Ile-----	-----Ala-----Ser-----
Z3	-----Gly-----	Asp	LysAsnCys-----ThrSer-----
Z321	GlyThrSer-----Asn-----	Lys	IleAspThr-----
JY1	-----Ser	Thr-----	AsnAsnAspThr-----Leu-----Ser-----

SUBSTITUTE SHEET

FIG. 2-8C

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FIG. 2-9A

	D4 ←	→ C4	
		*	
HXB2	GlySerAspThr.....IleThrLeuProCysArgIleLysGlnIleIleAsnMet		426
BRU	-----		431
MN	Gln-----Lys-----	426
SC	---Asn-----		426
SF2	---Asn-----Ile-----	-----Glu-----	426
NY5	AsnAsnGlu-----IleIle-----		423
CDC4	GlnLys-----GlyAspIle-----	-----Ser-----	418
WMJ2Ser-----Leu-----	-----Arg-----	435
RF	---Asn-----	-----Val-----	416
MAL	SerThrGlySer-----		433
ELI	AsnThrAsn....	Gln-----Lys-----	426
Z6	SerAspAsnLysLeu-----		424
Z3	SerAsnCys-----GlyAsn-----	-----ValValArgThr-----	425
Z321	ValAsn-----Ile-----	-----Val-----	426
JY1	---Thr.....	-----Lys-----	433

SUBSTITUTE SHEET

	CD4 binding region	*	
HXB2	TrpGlnLysValGlyLysAlaMetTyrAlaProIleSerGlyGlnIleArgCysSer		446
BRU	-----Glu-----		451
MN	-----Glu-----Glu-----		446
SC	-----Glu-----Lys-----ValLys-----		440
SF2	-----Glu-----Gly-----Ser-----		443
NY5	GlyArg-----+++		422
CDC4	-----Val-----Leu-----Lys-----Leu-----		455
WMJ2	-----Gly-----Gln-----		436
RF	-----Glu-----Lys-----Lys-----Ile-----		453
MAL	-----Thr-----Ala-----Val-----Asn-----Leu-----		446
ELI	ValAlaGlyArg...Ile-----GluArgAsn-----Leu-----		443
Z6	-----Gly-----Glu-----Asn-----		445
Z3	-----Gly-----Gln-----Glu-----Thr-----		440
Z321	-----Arg-----Gln-----Lys-----Val-----Val-----		446
JY1	-----Gly-----Glu-----Leu-----Lys-----Thr-----		453

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FIG. 2-9B

SUBSTITUTE SHEET

	[C4	→	D5	
HXB2	SerAsnIleThrGlyLeuLeuLeuThrArgAspGlyGly.....	AsnSer			461
BRU	-----	-----	-----	-----	466
MN	-----	-----	LysAspThrAsp	-----	463
SC	-----	-----	AsnSerLysAsnGlySerLys	-----	460
SF2	-----	-----	Thr-----Val	-----	459
NY5	-----	-----	-----	-----	
CDC4	-----	Gly-----Glu	-----	470
WMJ2	-----	AsnSer-----	-----	451
RF	-----	-----	GluAspThr	-----	469
MAL	-----	Ile-----	SerAsp	-----	463
ELI	-----	-----	IleAsn	-----	458
Z6	-----	-----	ThrAsn	-----	460
Z3	-----	AsnGlyLysCysAsnSerLys	-----	-----	460
Z321	Ile-----	ValGly-----Asn	-----	461
JY1	-----	Val-----Asn	-----	468

SUBSTITUTE SHEET

FIG. 2-9C

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HXB2	Asn	Asn	Glu	Ser	Glu	Ile	Phe	Arg	Leu	Gly	Gly	Gly	Asp	Met	Arg	Asp	Asn	Trp	Arg	Ser	481
BRU	---	Gly	---	---	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	---	---	486
MN	Thr	---	Asp	Thr	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	---	---	483
SC	---	Glu	Asn	Thr	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	---	---	480
SF2	Thr	---	Asp	Thr	---	Val	---	---	---	---	---	---	---	---	---	---	---	---	---	---	479
NY5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
CDC4	---	Gln	Thr	Thr	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	---	---	490
WMJ2	Ser	Ser	Arg	Glu	---	---	Pro	---	---	Asn	---	---	---	---	---	---	---	---	---	---	471
RF	Thr	---	Thr	Thr	---	---	---	---	---	Asn	---	---	---	---	---	---	---	---	---	---	489
MAL	---	Ser	Asp	Asn	---	Thr	Leu	---	Pro	---	---	---	---	---	---	---	---	---	Ile	---	483
ELI	---	Ser	Thr	Asn	---	Thr	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	478
Z6	---	Ser	Ser	Asn	---	Thr	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	480
Z3	+++	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	461
Z321	---	Thr	Ser	Asn	---	Thr	---	---	Pro	---	---	---	---	---	---	---	---	---	---	---	481
JY1	---	Ser	Thr	Asn	---	Thr	---	---	Pro	---	Lys	---	---	---	---	---	---	---	Asn	---	488

SUBSTITUTE SHEET

FIG. 2-10A

29 / 59

HXB2	GluLeuTyrLysTyrLysValValLysIleGluProLeuGlyValAlaProThrLysAla	501
BRU	-----	506
MN	-----Thr-----	503
SC	-----	500
SF2	-----Ile-----Ile-----	499
NY5	-----	
CDC4	-----	510
WMJ2	-----Arg-----	491
RF	-----Arg-----Arg-----	509
MAL	-----Arg-----	503
ELI	-----Gln-----Arg-----	498
Z6	-----	500
Z3	-----	
Z321	-----	501
JY1	-----Arg-----Ile-----Arg-----	508

SUBSTITUTE SHEET

FIG. 2-10B

	gp120 C5	gp160 C5 (TO END)	
	gp120 V gp41		
HXB2	LysArgArgValValGlnArgGluLysArgAlaValGly...IleGlyAlaLeuPheLeu		520
BRU	-----		525
MN	-----	Ala...	521
SC	-----	Thr-----Met-----	520
SF2	-----	IleVal-----Met-----	519
NY5	-----		
CDC4	-----	MetLeu-----Met-----	530
WMJ2	-----	Thr-----Met-----	511
RF	-----	Thr-----Met-----	529
MAL	-----Glu-----	Ile-----Leu-----Met-----	522
ELI	-----Glu-----	Ile-----Leu-----Met-----	517
Z6	-----Glu-----	Ile-----Leu-----Met-----	519
Z3	-----		
Z321	-----Ala-----	Ile-----Met...-----Phe-----	520
JY1	-----Glu-----	Ile-----Leu-----Val-----	527

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SUBSTITUTE SHEET

FIG. 2-10C

31 / 59

HXB2	GlyPheLeuGlyAlaAlaGlySerThrMetGlyAlaAlaSerMetThrLeuThrValGln	540
BRU	-----Arg-----	545
MN	-----Val-----	541
SC	-----Thr-----	540
SF2	-----Val-----Leu-----	539
NY5		
CDC4	-----Thr-----Ala-----	550
WMJ2	-----Gly-----Leu-----	531
RF	-----Gly-----Ile-----	549
MAL	-----Leu-----	542
ELI	-----Arg-----Val-----	537
Z6	-----Val-----	539
Z3		
Z321	-----Ile-----	540
JY1	-----Val-----ValAla-----Gly-----	547

FIG. 2-IIA

SUBSTITUTE SHEET

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HXB2	AlaArgGlnLeuLeuSerGlyIleValGlnGlnGlnAsnAsnLeuLeuArgAlaIleGlu	560
BRU	-----	565
MN	-----Leu-----	561
SC	-----Leu-----	560
SF2	-----	559
NY5	-----	
CDC4	-----Lys-----	570
WMJ2	-----Asp-----	551
RF	-----His-----	569
MAL	-----	562
ELI	-----Met-----	557
Z6	-----Met-----	559
Z3	-----	
Z321	-----Arg-----	560
JY1	-----	567

FIG. 2-IIB

SUBSTITUTE SHEET

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HXB2	AlaGlnGlnHisLeuLeuGlnLeuThrValTrpGlyIleLeuGlnLeuGlnAlaArgIle	580
BRU	-----	585
MN	-----Met-----Val	581
SC	-----	580
SF2	-----Val	579
NY5	-----Val	
CDC4	-----	590
WMJ2	-----Val	571
RF	-----Val	589
MAL	-----Val	582
ELI	-----	577
Z6	-----	579
Z3	-----	
Z321	-----Lys-----	580
JY1	-----Met-----Val	587

SUBSTITUTE SHEET

FIG. 2-IIIC

34 / 59

HXB2	LeuAlaValGluArgTyrLeuLysAspGlnGlnLeuLeuGlyIleTrpGlyCysSerGly	600
BRU	-----	605
MN	-----Phe-----	601
SC	-----Arg-----	600
SF2	-----Arg-----	599
NY5	-----	
CDC4	-----Phe-----	610
WMJ2	-----Arg-----	591
RF	-----Arg-----	609
MAL	-----Gln-----Arg-----Met-----	602
ELI	-----	597
Z6	-----	599
Z3	-----	
Z321	-----	600
JY1	-----Ser-----	607

SUBSTITUTE SHEET

FIG. 2-12A

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	*	[]	[]	
HXB2	LysLeuIleCysThrThrAlaValProTrpAsnAlaSerTrpSerAsnLysSerLeuGlu					620
BRU	-----					625
MN	-----Thr-----					621
SC	-----Thr-----Thr-----					620
SF2	-----					619
NY5	-----					
CDC4	-----Thr-----					630
WMJ2	-----Thr-----					611
RF	-----Thr-----					
MAL	-----His-----Phe-----Ser-----Arg-----Asn					629
ELI	-----His-----Asn-----Ser-----Arg-----Asn					622
Z6	-----Thr-----Ser-----					617
Z3	-----Thr-----Arg-----					619
Z321	-----Ile-----Pro-----Asn-----					620
JY1	-----His-----Thr-----Ser-----					627

SUBSTITUTE SHEET

FIG. 2-12 B

[illegible]

SUBSTITUTE SHEET

FIG. 2-12C

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HXB2	LeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLysAsnGluGlnGluLeu	660
BRU	-----	665
MN	-----Tyr-----Leu-----Lys-----Thr-----	661
SC	-----TyrThr-----	660
SF2	Thr-----TyrThr-----Leu-----	659
NY5	-----	
CDC4	-----TyrThr-----	670
WMJ2	Ile-----Tyr-----Gly-----Gln-----	651
RF	Ile-----TyrAsn-----Leu-----	669
MAL	Ile-----TyrAsn-----Ile-----Lys-----	662
ELI	-----Tyr-----Thr-----Lys-----	657
Z6	-----TyrArg-----Thr-----	659
Z3	-----	
Z321	Val-----TyrAsn-----Thr-----Ile-----ArgAsp-----	660
JY1	Val-----Tyr-----Asn-----Ile-----Asp-----	667

FIG. 2-13A

SUBSTITUTE SHEET

	[]	
HXB2	LeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPheAsnIleThrAsnTrpLeuTrp	680
BRU	-----	685
MN	-----Asp-----	681
SC	-----	680
SF2	-----Ser-----	679
NY5		
CDC4	Gln-----Thr----SerAsp-----Lys-	690
WMJ2	-----Asp-----	671
RF	-----Asn-----Asp-----Gln-----	689
MAL	-----Ser----SerLys-----	682
ELI	-----Ser-----Gln-----	677
Z6	-----Gln-----	679
Z3		
Z3Z1	---Ala-----Asn-----Asp---Ser-----	680
JY1	---Gln-----Ser----Lys-----	687

SUBSTITUTE SHEET

FIG. 2-13B

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HXB2	TyrIleLysLeuPheIleMetIleValGlyGlyLeuValGlyLeuArgIleValPheAla	700
BRU	-----Ile-----	705
MN	-----Ile-----	701
SC	-----Ile-----Thr	700
SF2	-----Ile-----	699
NY5		
CDC4	-----Ile-----Ile-----	710
WMJ2	-----Ile-----Ile-----Thr	691
RF	-----ArgIle-----Lys-----	709
MAL	-----ArgIle-----IleVal-----Ile-----	702
ELI	-----Ile-----Ile-----Ile-----	697
Z6	-----Ile-----Ile-----	699
Z3		
Z321	-----Ile-----Ile-----	700
JY1	-----Ile-----Ile-----Thr	707

FIG. 2-13C

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HXB2	ValLeuSerIleValAsnArgValArgGlnGlyTyrSerProLeuSerPheGlnThrHis	720
BRU	-----	725
MN	-----Leu-----Arg	721
SC	-----Arg	720
SF2	-----Arg	719
NY5	-----	
CDC4	-----Leu	730
WMJ2	-----	711
RF	-----	729
MAL	-----Leu-----Leu	722
ELI	-----Leu	717
Z6	-----Leu	719
Z3	-----	
Z321	-----Ile-----Leu	720
JY1	-----Leu-----Leu	727

FIG. 2-14A

SUBSTITUTE SHEET

← tat cds end

HXB2	LeuProIleProArgGlyPro...AspArgProGluGlyIleGluGluGlyGlyGlu	739
BRU	-----Thr-----	744
MN	Pro-----Val-----	748
SC	-----SerGln-----	739
SF2	-----Val-----Asp-----	738
NY5		
CDC4	-----Asn-----Thr-----Gly-----	749
WMJ2	-----Thr-----	738
RF	-----Ala-----Gly-----	748
MAL	-----Thr-----Pro-----	742
ELI	-----Ala-----Thr-----	736
Z6	-----Ala-----Glu-----	738
Z3		
Z321	ThrHisHisGln-----Glu-----Arg-----Gly-----	739
JY1	-----Ala-----	746

SUBSTITUTE SHEET

FIG. 2-14B

HXB2	ArgAspArgAspArgSerIleArgLeuValAsnGlySerLeuAlaLeuIleTrpAspAsp	759
BRU	-----	764
MN	-----Thr-----Gly-----His-----Phe-----Ile-----Val-----	768
SC	-----Gly-----Gly-----Asp-----Phe-----Ile-----Val-----	759
SF2	-----Val-----Val-----Asp-----Phe-----Glu-----	758
NY5	-----	
CDC4	-----Gly-----Gly-----Thr-----His-----Phe-----Val-----	769
WMJ2	-----Val-----Val-----His-----Phe-----	758
RF	-----GlyGlyAla-----Phe-----Thr-----	768
MAL	GlnGly-----Gly-----PheSer-----	762
ELI	-----Gly-----Val-----Leu-----PheSer-----	756
Z6	-----Gly-----PheSer-----	758
Z3	-----	
Z321	Gln-----Ser-----Phe-----Pro-----Ala-----	759
JY1	GlnGly-----PheSer-----Phe-----	766

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FIG. 2-14C

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HXB2	LeuArgSerLeuCysLeuPheSerTyrHisArgLeuArgAspLeuLeuIleValThr	779
BRU	-----	784
MN	-----Phe-----His...-----AlaAla	779
SC	+++-----	779
SF2	-----Arg-----AlaAla	778
NY5	-----	
CDC4	-----Ala	789
WMJ2	-----Lys	770
RF	---TrpThr---Ser---Val	788
MAL	---Asn---Ala	782
ELI	-----Ile-----AlaVal	776
Z6	---Asn---Ile---AlaAla	778
Z3	-----	
Z321	-----Cys-----CysAla-----AlaAla	779
JY1	---Asn---Ile---Ala	786

FIG. 2-15A

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HXB2	ArgIleValGluLeuLeuGlyArgArgGlyTrpGluAlaLeuLysTyrTrpTrpAsnLeu	799
BRU	-----	804
MN	-----Val-----	799
SC	-----	799
SF2	---Thr---Ile---His-----Ser---	798
NY5	-----	
CDC4	-----Val-----	809
WMJ2	-----	790
RF	-----	808
MAL	-----Leu-----	802
ELI	-----AspIle-----Leu-----	796
Z6	-----Leu-----	798
Z3	-----	
Z321	---Thr---Ile---Thr-----LeuGly---	799
JY1	-----Ile-----Leu---Ser---	806

FIG. 2-15B

SUBSTITUTE SHEET

← trs/art cds end []

HXB2	LeuGlnTyrTrpSerGlnGluLeuLysAsnSerAlaValSerLeuLeuAsnAlaThrAla	819
BRU	-----	824
MN	-----Ser-----	819
SC	-----Arg-----PheVal-----	819
SF2	-----Ile-----Trp-----	818
NY5	-----	
CDC4	-----Val-----Val-----Val-----	829
WMJ2	-----Lys-----Gly-----Ile-----	818
RF	-----Thr-----Thr-----	828
MAL	-----Gly-----Ile-----Thr-----	822
ELI	-----Arg-----Ser-----PheAsp-----Ile-----	816
Z6	-----Arg-----Arg-----Ser-----AspThrIle-----	818
Z3	-----	
Z321	ValIle-----Gly-----IleAsn-----AspThrVal-----	819
JY1	-----Thr-----PheIle-----	826

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FIG. 2-15C

HXB2	IleAlaValAlaGluGlyThrAspArgValIleGluValValGlnGlyAlaCysArgAla	839
BRU	-----	844
MN	-----Leu---Arg---Gly-----	839
SC	-----LeuLeu---Arg---Phe-----	839
SF2	-----Thr-----Ala---Arg---Tyr-----	838
NY5	-----	
CDC4	-----ArgIleTyr-----	849 ⁴⁶ / 59
WMJ2	-----ArgIle-----	838
RF	-----Ile-----Ala---ArgIleLeu-----	848
MAL	-----Cys-----IleGly---ArgPheGly-----	842
ELI	-----IleIle---Arg-----	836
Z6	-----Ile---ArgArgThrTyr-----	838
Z3	-----	
Z321	-----AspTrp-----Arg---Gly-----	839
JY1	-----Ile-----LeuIleArgArg---Phe-----	846

FIG. 2-16A

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HXB2	IleArgHisIleProArgArgIleArgGlnGlyLeuGluArgIleLeuLeu++	857
BRU	-----	862
MN	---Leu-----Thr-----Ala-----	857
SC	---Leu-----Thr-----Ala-----Gln-----	857
SF2	---Leu-----His-----Leu-----	856
NY5	-----	
CDC4	PheLeu-----Phe-----Ala-----	867
WMJ2	---Ile-----	848
RF	PheLeu-----	866
MAL	---Leu-----Phe-----Ala-----	860
ELI	ValLeuAsn-----	854
Z6	ValLeuAsnVal---Thr-----Leu-----	856
Z3	-----	
Z321	PheLeuAsn-----	857
JY1	ValLeu-----Val-----Ala-----	864

FIG. 2-16B

SUBSTITUTE SHEET

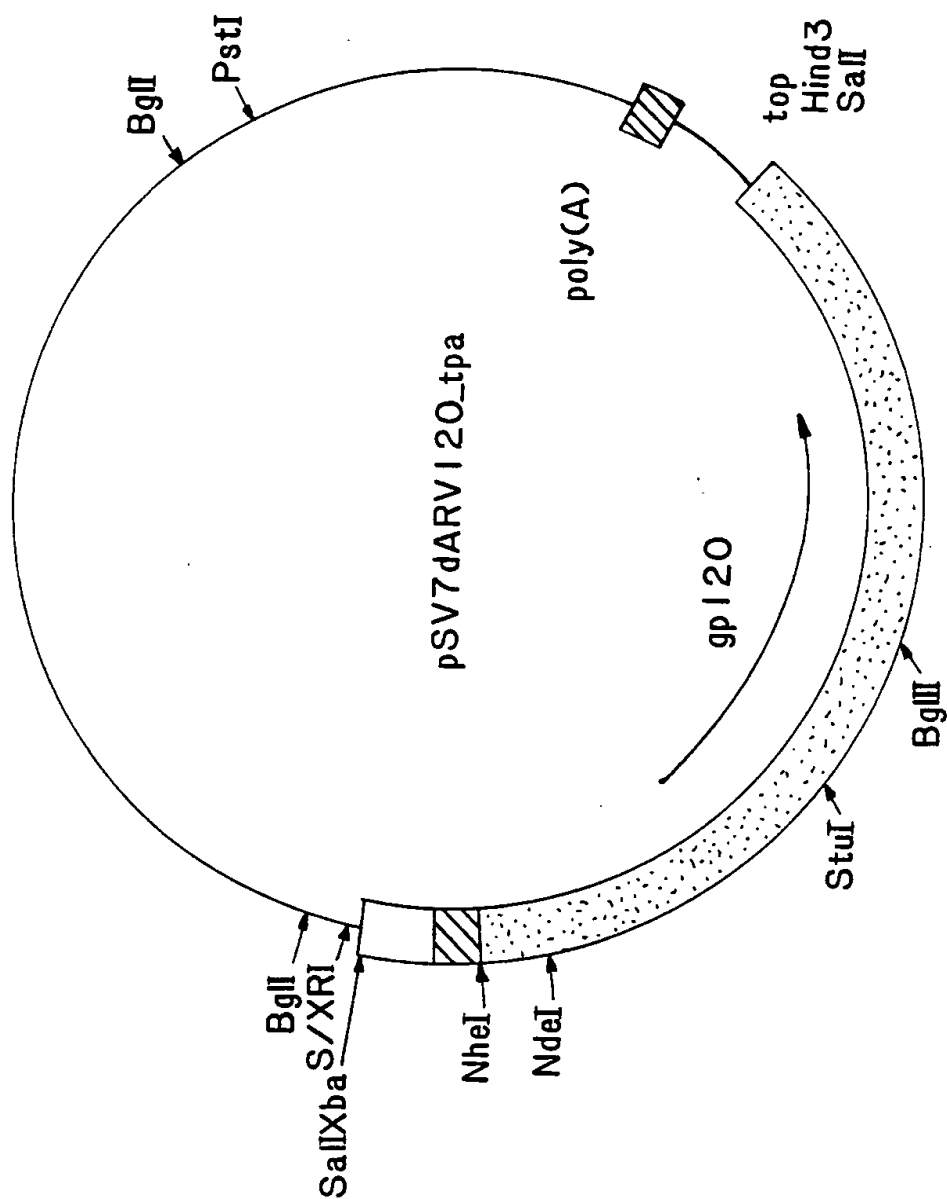


FIG. 3

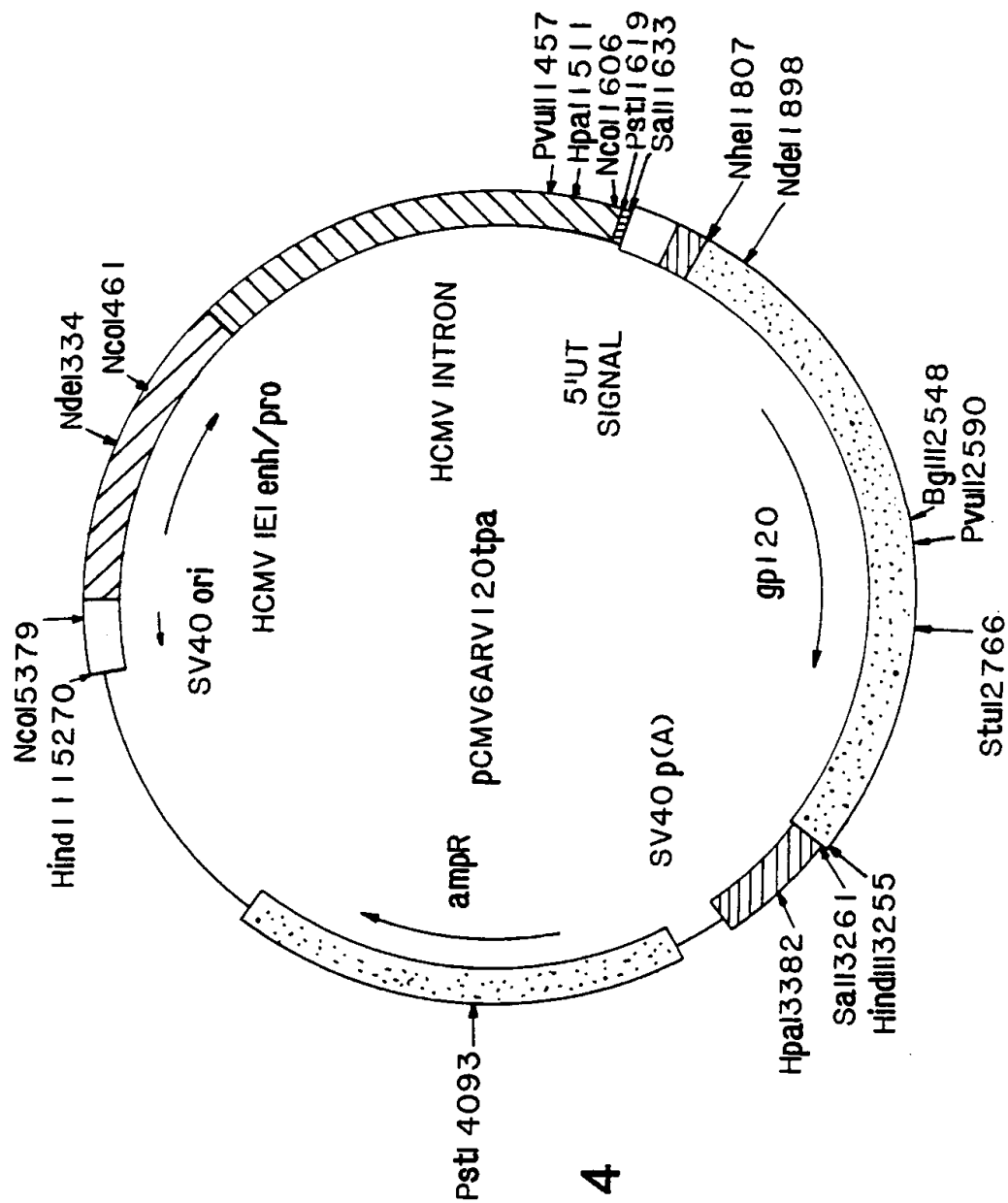


FIG. 4

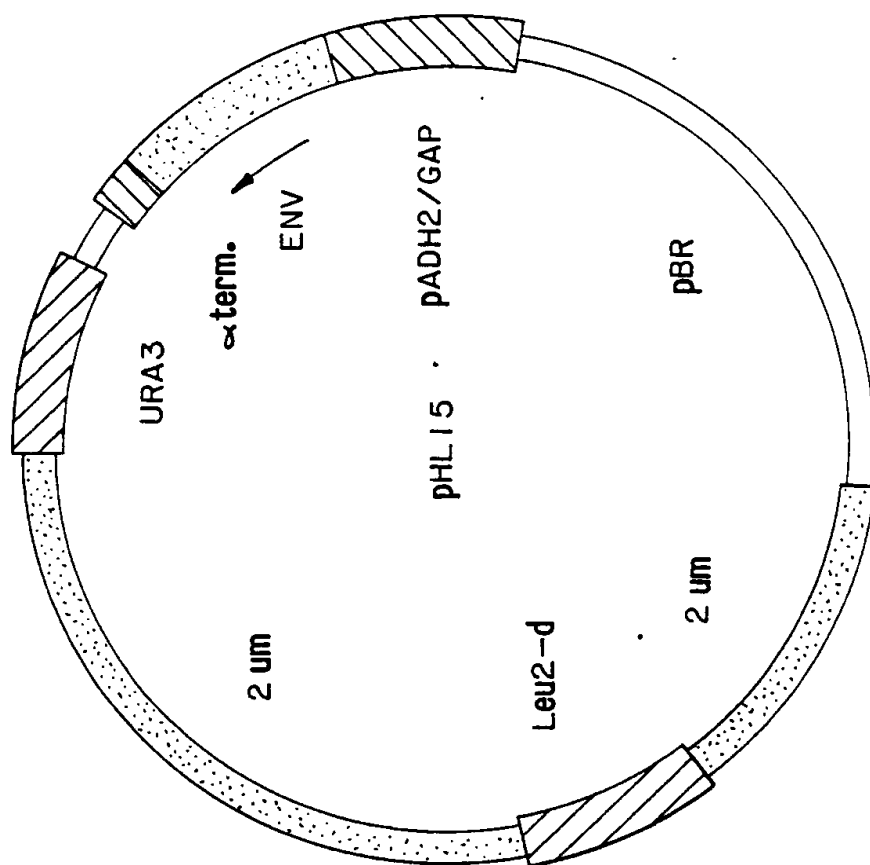


FIG. 5

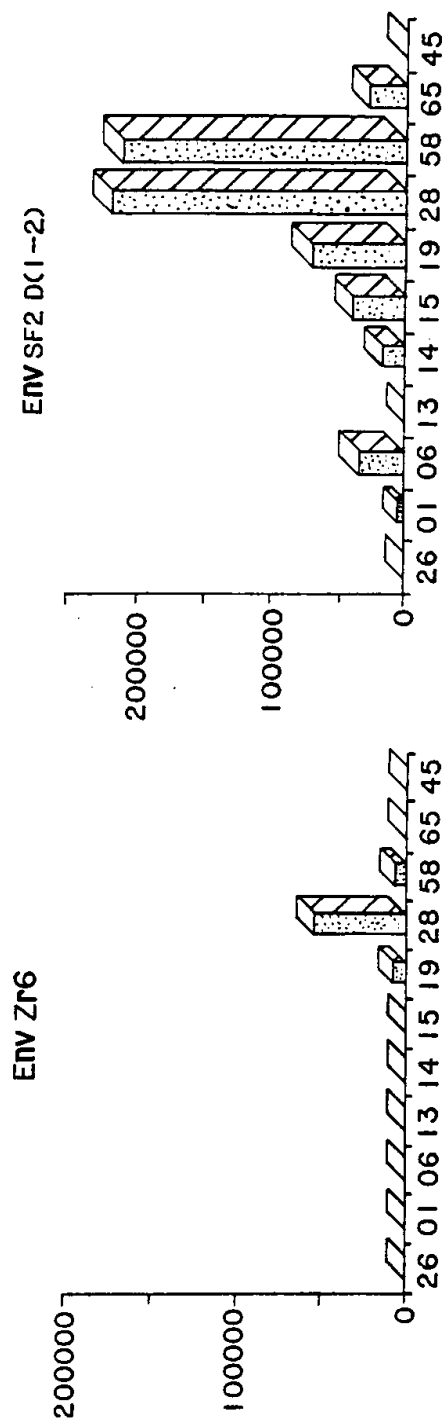
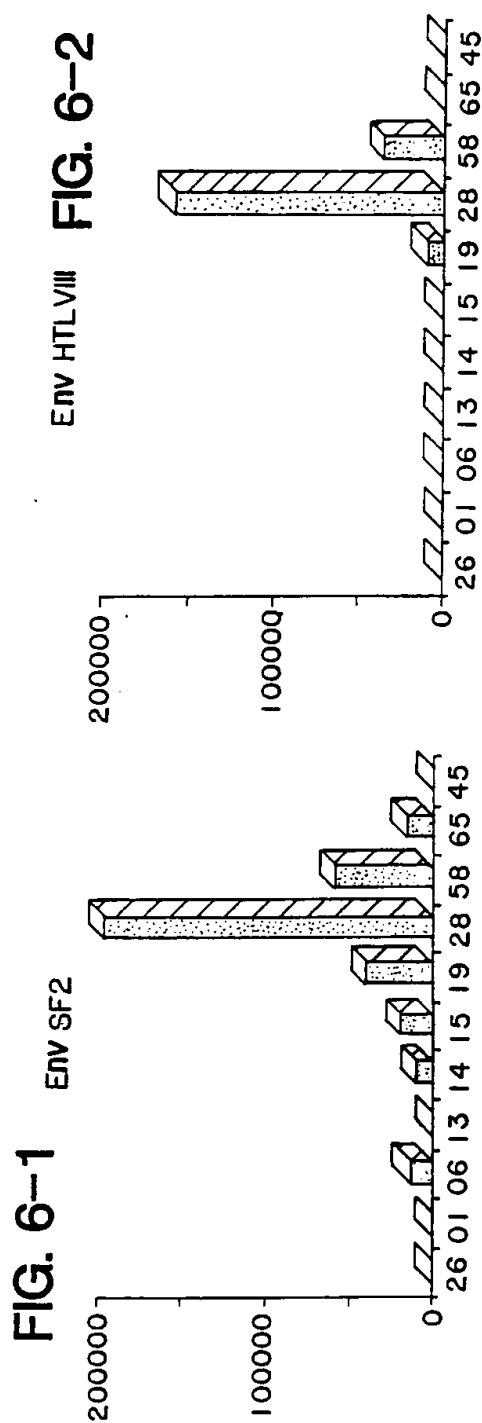


FIG. 6-6 ENV SF2 D(3-5)

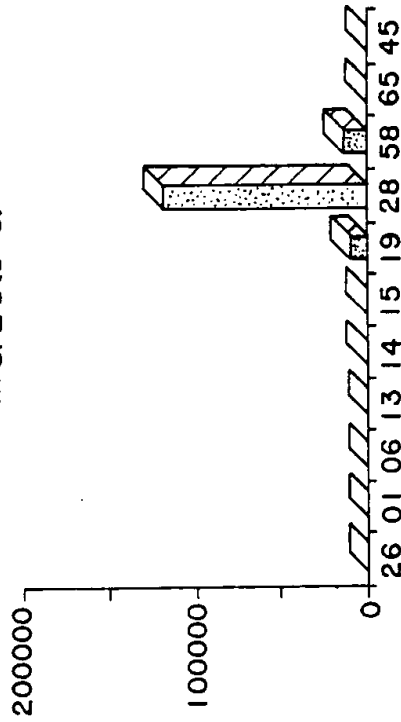


FIG. 6-5 ENV SF2 D3E

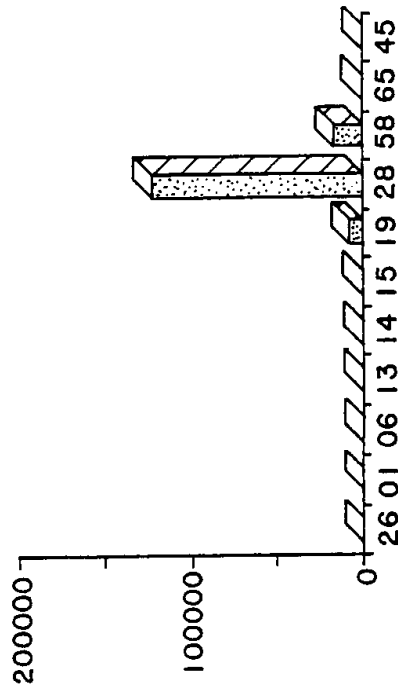
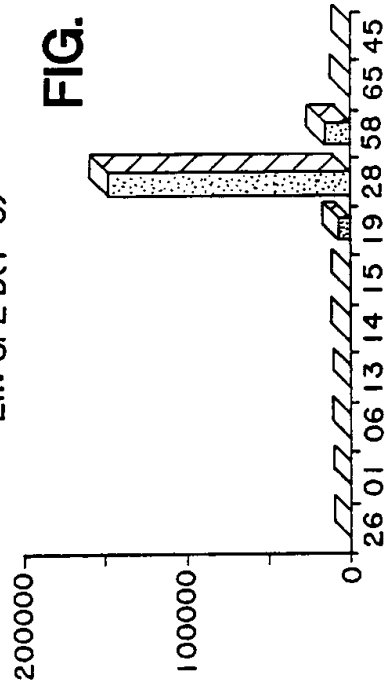
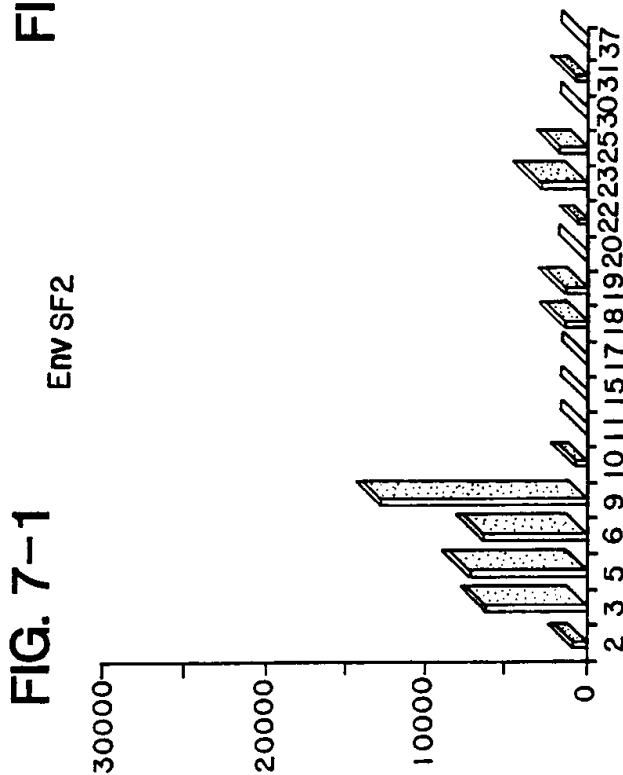
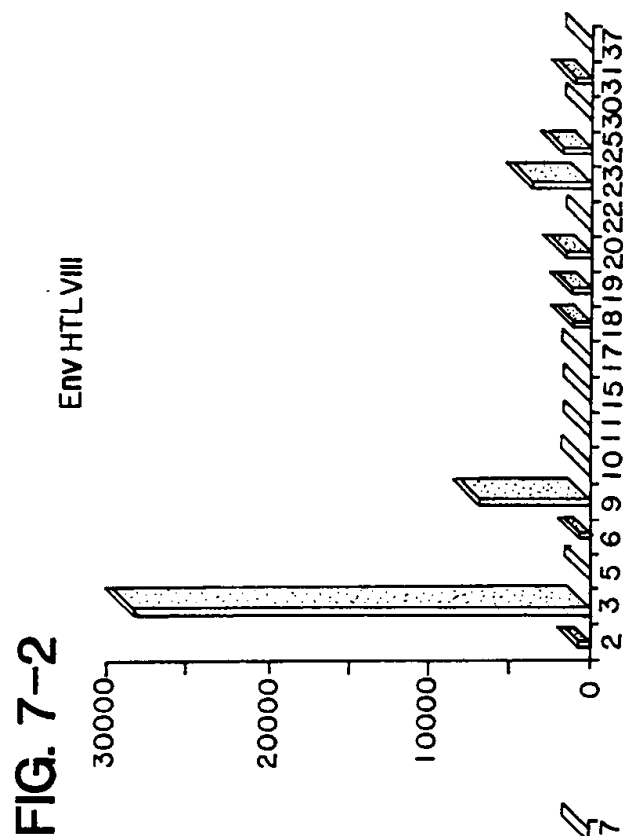


FIG. 6-7

ENV SF2 D(1-5)





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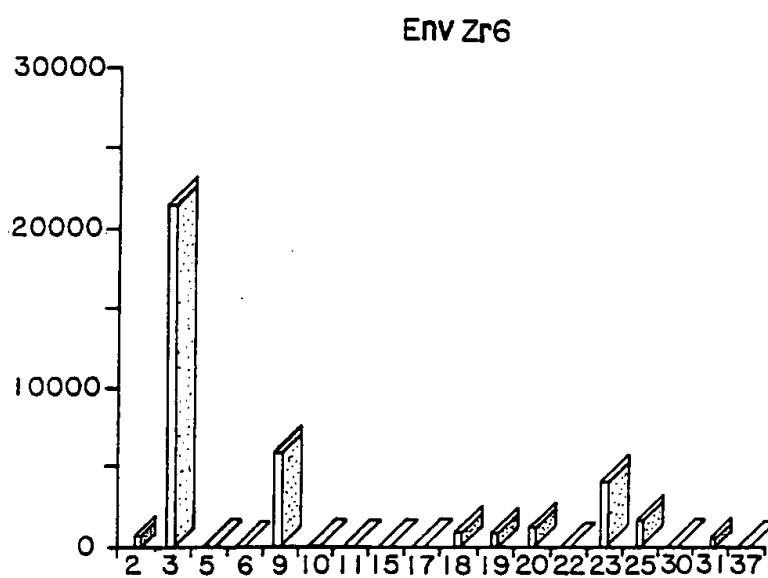


FIG. 7-3

SUBSTITUTE SHEET

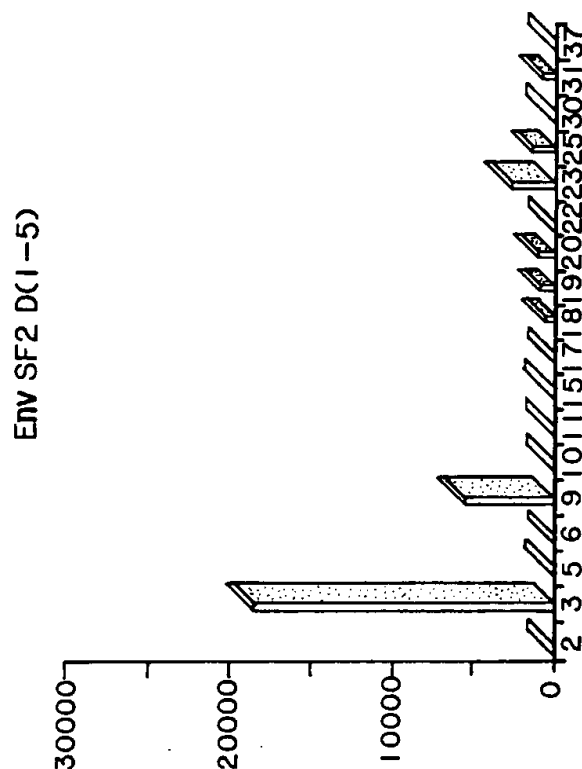


FIG. 7-5

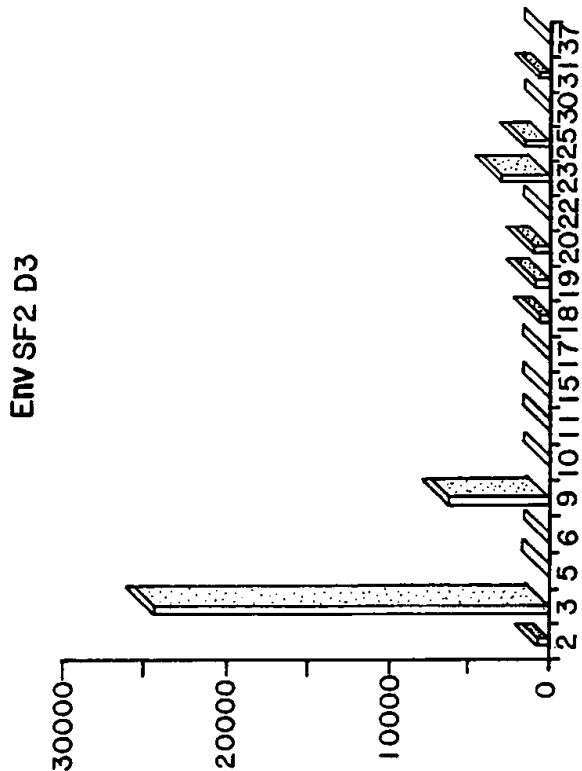


FIG. 7-4

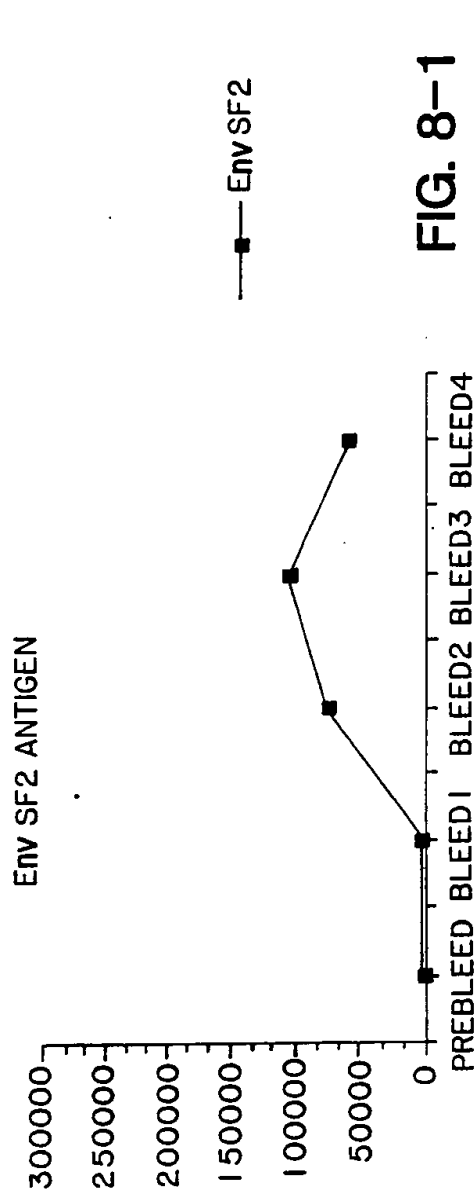


FIG. 8-1

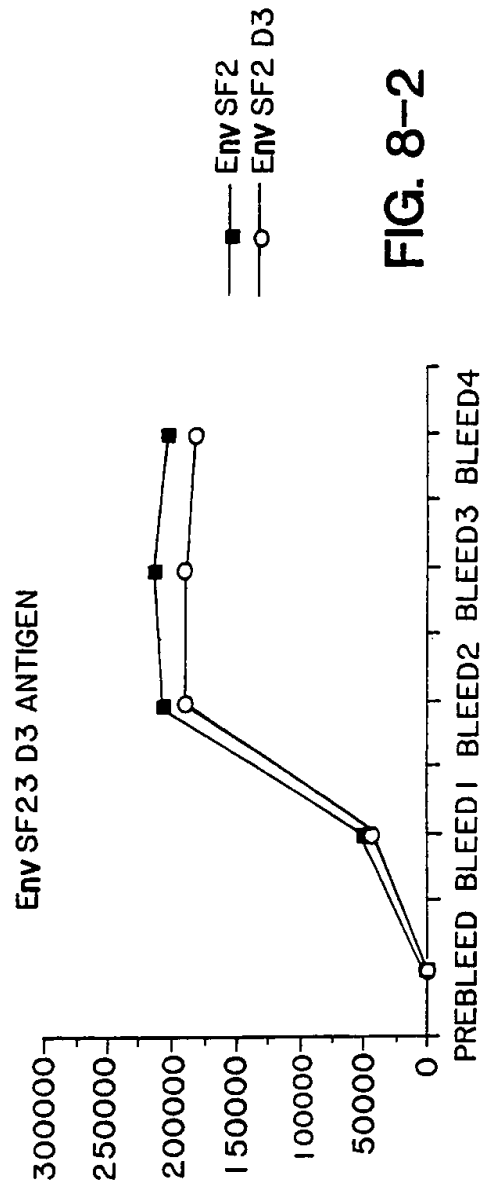


FIG. 8-2

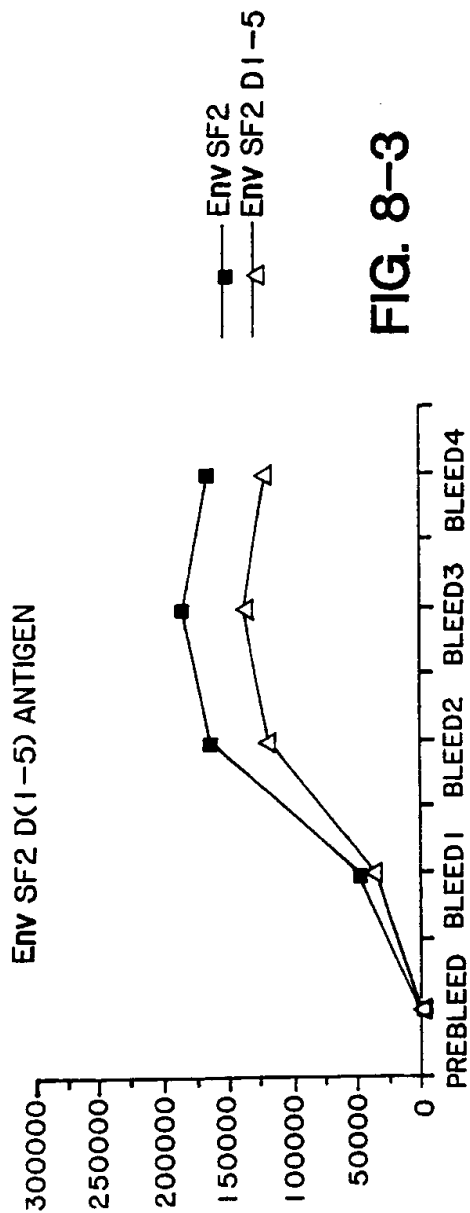


FIG. 8-3

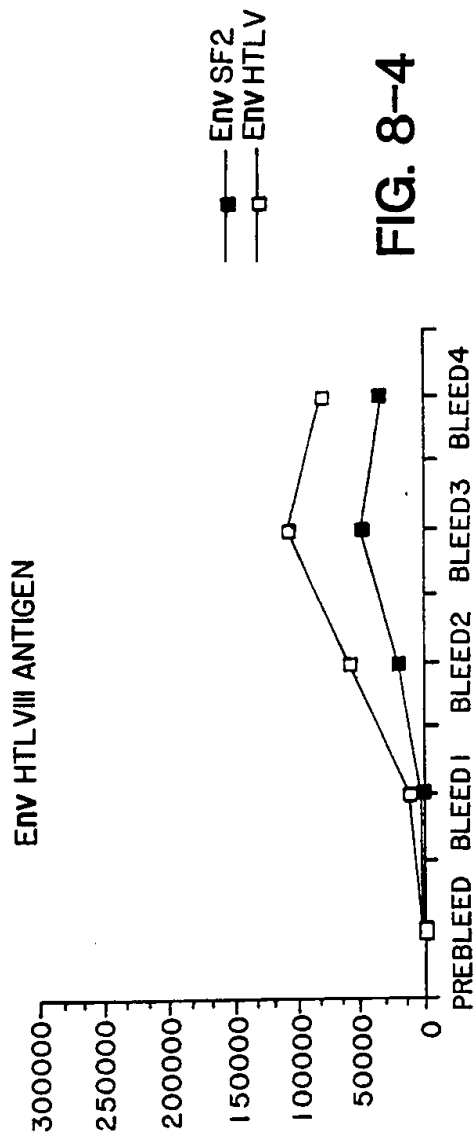


FIG. 8-4

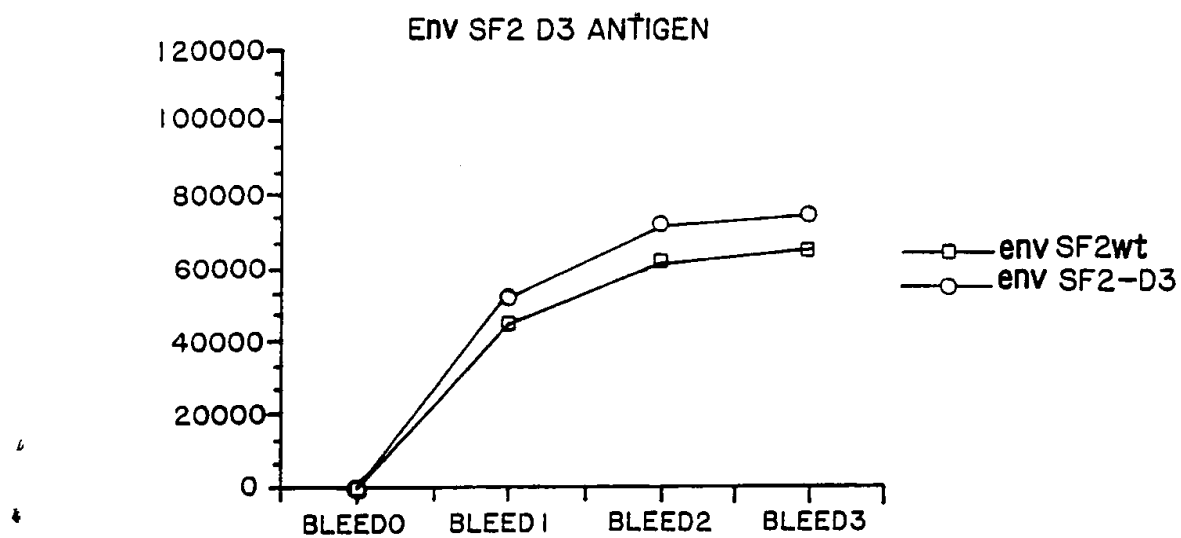
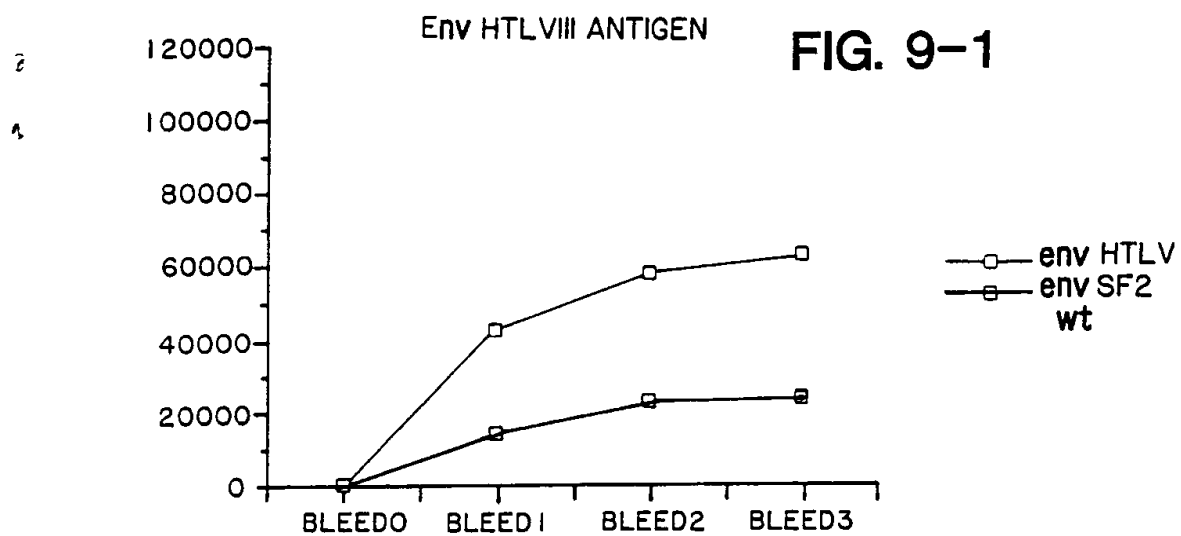


FIG. 9-2

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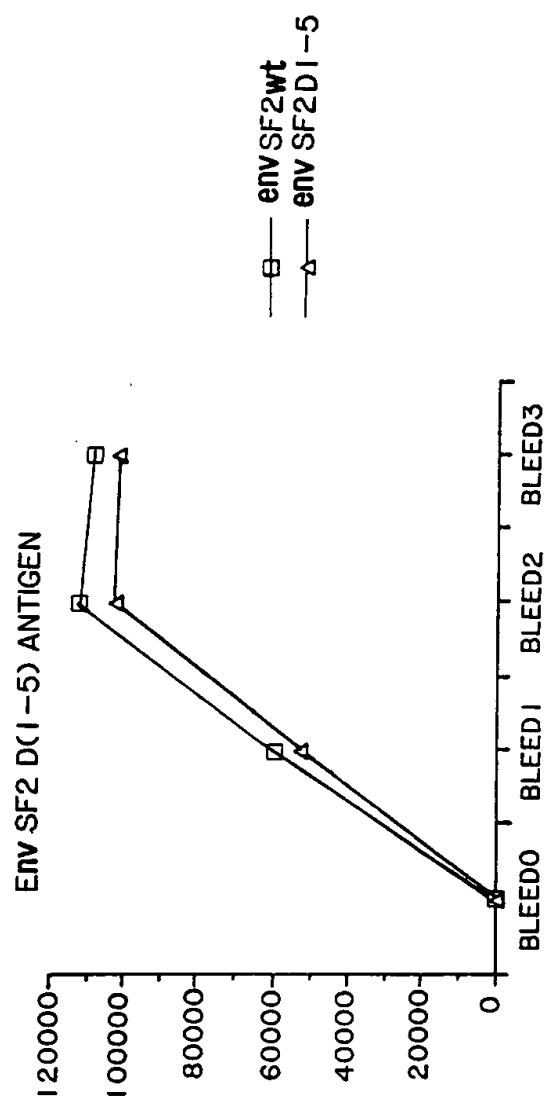


FIG. 9-3

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03605

I. CLASSIFICATION OF SUBJECT MATTER (In several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K39/395; C12P21/02; C12Q1/70; C12N15/00

U.S. Cl.: 530/326; 435/5,7; 424/85.8, 88, 89; 435/172.3, 320

II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System

Classification Symbols

U.S.

530/326; 435/5,7; 424/85.8, 88, 89; 435/172.3, 320

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched *

Databases: Chemical Abstracts Services Online (File CA, 1967-1989; File Biosis, 1969-1989). Automated Patent System (File USPAT, 1975-1989). Protein databases: PIR, Swiss-Prot.

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	EP 0187041, published 9 July 1986, (Capon), "Molecularly cloned acquired immunodeficiency syndrome polypeptides, intermediates therefor and methods and materials for their use."	1-35
X Y	Journal of Virology, Volume 61, No. 2, February 1987, (Modrow), "Computer-Assisted Analysis of Envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions", see pages 570-578.	1-26, 31-35 28-30
X Y	Vaccines 87, Cold Spring Harbor Laboratory, 1987, (Alizon), "Genetic variability of the human and simian aids virus", see pages 146-153.	1-30 31-35

* Special categories of cited documents: **

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

22 December 1989

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

22 JAN 1990

Signature of Authorized Officer

D. Bernstein

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE:

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____ because they relate to subject matter ¹³ not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹⁴, specifically:

3. ☐ Claim numbers _____ because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING:

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant; ~~Consequently, this international search report covers only the claims for which fees were paid.~~ however, the International Searching Authority has decided to search all groups corresponding to claims 1-35.
4. ☒ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority ~~has~~ ^{has} therefore searched all groups that correspond to claims 1-35.
Remark on Protest
 - ☐ The additional search fees were accompanied by applicant's protest.
 - ☐ No protest accompanied the payment of additional search fees.